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Sphingosine-1-phosphate (SPP), a bioactive sphingolipid metabolite, inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 human breast cancer cell line. Similar to many other cell types, SPP stimulated proliferation of MDA-MB-231 cells, albeit to a lesser extent. Treatment of MDA-MB-231 cells with SPP had no significant effect on their adhesiveness to Matrigel, and only high concentrations of SPP partially inhibited matrix metalloproteinase 2 activation induced by Con A. However, SPP at a concentration that strongly inhibited invasiveness, also markedly reduced chemotactic motility. To investigate the molecular mechanisms by which SPP interferes with cell motility, we examined tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, which are important for organization of focal adhesions and cell motility. SPP rapidly increased tyrosine phosphorylation of FAK and paxillin and of the paxillin-associated protein Crk. Overexpression of FAK and kinase-defective FAK in MDA-MB-231 cells resulted in a slight increase in motility without affecting the inhibitory effect of SPP, whereas expression of FAK with a mutation of the major autophosphorylation site (F397) abolished the inhibitory effect of SPP on cell motility. In contrast, the phosphoinositide 3'-kinase inhibitor, wortmannin, inhibited chemotactic motility in both vector and FAK-F397 transfected cells. Our results suggest that autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to decreased cell motility. Since SPP has been implicated as a lipid signaling molecule with novel dual intra and intercellular actions, it was of interest to determine whether the effect of SPP on chemotactic motility of human breast cancer cells is mediated intracellularly or through the recently identified EDG family of G protein-coupled SPP receptors. In contrast to SPP, dihydro-SPP, which binds to and signals through SPP receptors, EDG-1, -3, and –5, had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. Caged SPP inhibited chemotactic motility of MDA-MB-231 cells only upon ultraviolet irradiation. In addition, in MCF-7 cells, overexpression of sphingosine kinase, the enzyme that produces SPP, inhibited chemotactic motility compared to vector-transfected cells, and markedly increased cellular SPP levels, in the absence of detectable secretion. Our results suggest that the inhibitory effect of SPP on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of SPP rather than through cell surface receptors.

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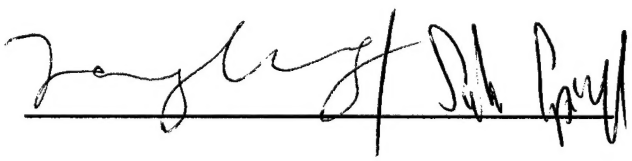

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INTRODUCTION

Metastasis is a multifactorial process by which tumor cells escape from the primary tumor, disseminate through blood and lymph vessels, evade host immune defense and home to specific target organs where they extravasate and re-colonize. Cell movement is considered a crucial step for the invasion of cancer cells (Lester and McCarthy 1992; Mueller et al. 1992). For cellular locomotion to occur, the cell must be able to protrude, form and break adhesion sites between the cell and the substratum, and move the cell mass. The assembly of focal adhesions, structures that link actin filaments and integrin-associated points of attachment with the extracellular matrix (Craig and Johnson 1996), and the concomitant recruitment of actin stress fibers and other cytoskeletal proteins is important for cell migration (Stossel 1989; Clark and Brugge 1995). Increased tyrosine phosphorylation of several proteins which are associated with focal adhesions has been implicated in cell migration and invasion (Mueller et al. 1992; Clark and Brugge 1995). Among them, focal adhesion kinase (FAK), is a widely expressed and highly conserved non-receptor protein tyrosine kinase that has been implicated in organization of focal adhesions (Schaller et al. 1992), formation of actin stress fibers (Seufferlein and Rozengurt 1994; Abedi and Zachary 1997) and regulation of motility (Cary et al. 1996; Carloni et al. 1997; Cary et al. 1998) and invasion (Scott and Liang 1995; Tremblay et al. 1996). FAK, in turn, phosphorylates and/or associates with other cytoskeletal components, including Src, the Crk-associated substrate, p130Cas (Cas), paxillin and tensin (Clark and Brugge 1995; Hanks and Polte 1997), that will either lead to activation of signaling pathways or establish contacts with talin, vinculin,__-actinin,and the actin polymers, essential components of stress fibers regulation.

Recent studies indicate that the sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate (SPP) can enhance tyrosine phosphorylation of FAK leading to stress fiber formation and focal contact assembly in Swiss 3T3 cells (Seufferlein and Rozengurt 1994; Wang et al. 1997). Moreover, in human arterial smooth muscle cells, SPP generated in response to PDGF, interfered with the dynamics of PDGF-stimulated actin filament disassembly and assembly resulting in a marked inhibition of cell spreading, extension of the leading lamellae, and of chemotaxis toward PDGF (Bornfeldt et al. 1995). These results suggest that endogenous SPP may play an important role in

regulating cell migration and chemotactic signaling. In contrast, other studies suggest that SPP acts through a cell surface receptor to inhibit motility (Yamamura et al. 1997) and induce cell rounding (Postma et al. 1996). At very low concentrations (10 nM), SPP, but not sphingosine, effectively inhibited chemotactic motility of several tumor cell lines, including mouse and human melanoma, and human osteosarcoma cells, whereas the motility of endothelial cells was not affected (Sadahira et al. 1992; Yamamura et al. 1997). However, the mechanism by which SPP inhibits motility is still not understood. In this study, we examined the effects of SPP on invasiveness and motility of the highly invasive MDA-MB-231 human breast cancer cell line, and also examined the involvement of FAK.

BODY

A. INVOLVEMENT OF FOCAL ADHESION KINASE IN INHIBITION OF MOTILITY OF HUMAN BREAST CANCER CELLS BY SPP

SPP inhibits chemoinvasiveness of MDA-MB-231 cells. To examine whether sphingolipid metabolites affect invasion, MDA-MB-231 cells were pretreated with SPP, sphingosine, cell permeable ceramide analogs (C2-Cer and C6-Cer), or sphingomyelinase and then chemoinvasion towards fibroblast conditioned medium was measured. A significant inhibitory effect was found at 5 μM SPP and a maximal effect was observed at 10-20 μM. Sphingosine, at 20 μM, has a smaller inhibitory effect. Similarly, SPP inhibits chemoinvasion towards fetal bovine serum (FBS). In contrast, sphingosine and SPP were not cytotoxic up to 20 and 50 μM, respectively. Similar results were obtained when cells were treated with sphingosine or SPP for only 4 h during the chemoinvasion towards fibroblast conditioned medium and FBS (Fig. 1B in Appendix 1). However, the inhibitory effects required higher concentrations when incubated with the cells for 4 h rather than 24 h.

Effects of sphingosine-1-phosphate on proliferation of MDA-MB-231 cells. SPP is a potent mitogen for many different cell types (Spiegel and Milstien 1995) and also induced a moderate increase in DNA synthesis of MDA-MB-231 (Fig. 2 A in Appendix 1) and MCF-7 cells. Consistent with these results, flow cytometric analysis of cell cycle distribution revealed that optimally mitogenic

concentrations of SPP (10 μM) induced a slight increase in the proportion of MDA-MB-231 cells in S phase, with a concomitant decrease in the proportion of cells in Go-G1 (Fig. 2 B in Appendix 1).

SPP inhibits MMP-2 activation and has no effect on adhesion of MDA-MB-231 cells to Matrigel. To determine whether the effect of SPP on invasion was due to altered adhesion, cells were pretreated with SPP or anti-β1 integrin antibody (20 μg/ml) for 24 h and adherence to Matrigel was determined (Fig. 3 A in Appendix 1). Treatment of MDA-MB-231 cells with SPP at concentrations that strongly inhibited invasiveness (5-10 μM) had no significant effects on the adhesiveness of cells to Matrigel. As expected, pretreatment of cells with anti-integrin β1 antibody, strongly inhibited adhesion to Matrigel (Fig. 3A in Appendix 1), while treatment of cells with an unrelated antibody had no effect.

To penetrate the extracellular matrix, metastatic cells disrupt local segments of the basement membrane with proteinases, such as MMP-2 (Liotta 1986). In breast adenocarcinomas, MMP-2 is secreted by the reactive stroma and appears to be sequestered by the carcinoma cells (Noel et al. 1994). MMP-2 is secreted as a proenzyme (72 kD) whose proteolytic activity is triggered by truncation and conformational rearrangement at the cell surface (Stetler-Stevenson 1990). Due to the importance of activation of MMP-2 in matrix degradation in breast cancer (Azzam et al. 1993), the effect of SPP on MMP-2 activation by MDA-MB-231 cells was examined. In agreement with previous studies (Maiti et al. 1994; Yu et al. 1995), Con A induced activation of MMP-2 (Fig. 3B in Appendix 1). Whereas SPP alone did not significantly affect MMP-2 activation, it inhibited Con A-induced activation of MMP-2 only at high concentrations. Our results indicate that MMP-2 activation does not appear to be a major target of SPP in inhibition of chemoinvasion by these cells.

SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells. As previously reported, MDA-MB-231 cells are much more motile than the MCF-7 cells (cell number per field was 1720 ± 80 for control MDA-MB-231 and 520 ± 75 for MCF-7 cells). SPP markedly inhibited chemotaxis at 5-10 μM in both cell lines. Similar to the results of the chemoinvasion assays, SPP was more potent than sphingosine at inhibiting motility of MDA-MB-231 cells (Fig. 4A in Appendix 1).

Cells treated with SPP for 4 h in the chamber without pretreatment also showed decreased motility compared with untreated cells (Fig. 4C in Appendix 1). However, in this case, maximal inhibitory effects required higher concentrations of SPP.

SPP increases tyrosine phosphorylation of focal adhesion kinase. Previously we have shown that SPP stimulates tyrosine phosphorylation of FAK in quiescent Swiss 3T3 fibroblasts (Wang et al. 1997), whereas no effect on FAK phosphorylation could be detected in human arterial smooth muscle cells whose chemotactic mobility was markedly inhibited by SPP (Bornfeldt et al. 1995). To examine the effects of SPP on FAK phosphorylation in MDA-MB-231 cells, lysates were immunoprecipitated with anti-FAK mAb and analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody. SPP (10 μM) induced rapid tyrosine phosphorylation of FAK, reaching maximal levels within 5 min and declining thereafter (Fig. 5A in Appendix 1). Treatment of cells with 10 μM sphingosine also induced tyrosine phosphorylation of FAK (Fig. 5B in Appendix 1), whereas, the cell permeable ceramide analog C6-Cer had almost no effect (Fig. 5C in Appendix 1).

The major autophosphorylation site of FAK, Y397, is important for inhibition of motility of MDA-MB-231 cells by SPP. Recent studies demonstrate the importance of the major autophosphorylation site Y397 of FAK for CHO cell migration on fibronectin (Cary et al. 1996). To examine the role of FAK in MDA-MB-231 cells, we utilized these FAK constructs. After transfection and FACS isolation, cells expressing epitope-tagged WT, kinase-defective, or F397 FAK were screened by immunostaining with anti-KT3 antibody. A protein (125 kD) which was recognized by the KT3 antibody was detected in all three FAK transfected cell lines but not in control cells transfected with vector alone (Fig. 7A in Appendix 1). Total FAK expression was also increased after transfection as detected by western blotting with anti-FAK antibody (Fig. 7A in Appendix 1).

To determine the effects of FAK phosphorylation on cell migration, chemotactic motility of these cell lines was analyzed by Boyden chamber assays. In agreement with previous studies (Cary et al. 1996), the WT and KD cell lines exhibited slightly increased levels of migration compared to vector-transfected cells, whereas expression of F397 resulted in decreased motility. These results suggest that,

similar to other cell types, autophosphorylation of Y397 of FAK is necessary for FAK-dependent motility of MDA-MB-231 cells.

Because SPP increased tyrosine phosphorylation of FAK and its associated substrates, it was of interest to examine the involvement of FAK in SPP signaling leading to decreased cell motility. SPP markedly inhibited motility of cells overexpressing WT and KD FAK but not in cells expressing autophosphorylation site mutated FAK (Fig. 7B in Appendix 1). Moreover, the effect of SPP on WT cells was time-dependent. Inhibition of motility was proportional to the length of time that the cells were exposed to SPP. However, SPP did not have any effect on motility of F397 cells at the time period examined. It is possible that overexpression of F397 might cause MDA-MB-231 to become unresponsive to any motility inhibiting drugs. As shown in Fig. 7D in Appendix 1, wortmannin strongly inhibited chemotactic motility in both vector transfected and F397 cells. These results indicate that the motility of these cells is not generally compromised by overexpression of autophosphorylation site mutated FAK and suggest that Y397 is essential for SPP-induced inhibition of motility which is independent of the PI3-kinase pathway.

B. SPP INHIBITS MOTILITY OF HUMAN BREAST CANCER CELLS INDEPENDENTLY OF CELL SURFACE RECEPTORS

Previously, many studies have shown that exogenous SPP inhibits the chemotactic motility of various cancer cells at very low nanomolar concentrations (10-100 nM) (Sadahira et al. 1992; Sadahira et al. 1994; Kawa et al. 1997). SPP also inhibits integrin-dependent motility (haptotactic motility) of mouse melanoma B16 cells by inhibiting actin nucleation and pseudopodium formation, without reducing integrin-dependent adhesion to the extracellular matrix (Sadahira et al. 1994). Furthermore, SPP immobilized on controlled pore glass beads inhibits motility of mouse melanoma cells (Yamamura et al. 1997), indicating that this effect may be mediated through cell surface receptors. In contrast, we found that inhibition of chemotactic motility of human breast cancer MCF-7 and MDA-MB-231 cells requires micromolar concentrations of SPP (Wang et al. 1999). Thus, it is of interest to determine whether SPP acts extracellularly as a ligand for cell surface receptors or intracellularly as a second messenger to inhibit chemotactic motility of human breast cancer cells. In this work, we demonstrated

that motility of human breast cancer cells was inhibited when intracellular levels of SPP were increased either after photolysis of caged SPP or by overexpression of sphingosine kinase, both approaches bypass cell surface receptors. Our results suggest that SPP can inhibit motility through intracellular actions.

SPP Inhibits Chemotactic Motility of Human Breast Cancer Cells. In agreement with our previous report (Wang et al. 1999), we found that SPP inhibits chemotaxis of human breast cancer cells (Fig. 1A in Appendix 2). SPP (10 μM) decreased chemotaxis of estrogen-independent MDA-MB-231 and BT 549 human breast cancer cells by 45% and 28%, respectively. Treatment of estrogen-dependent human breast cancer cells with SPP decreased chemotaxis by 87% (MCF-7) and 45% (ZR-75-1). In addition, treatment with high μM concentrations of SPP and sphingosine also slightly, but significantly, inhibited random motility of MDA-MB-231 cells (Fig. 1B in Appendix 2).

Expression of SPP Receptors in MDA-MB-231 and MCF-7 Cells. It has recently been reported that SPP binds specifically and with high affinity to the GPCRs, EDG-1 (Lee et al. 1998), EDG-3 and EDG-5 (H218/AGR16) (Van Brocklyn et al. 1999). We therefore examined expression of SPP receptor mRNAs in MCF-7 and MDA-MB-231 cells using RT-PCR analysis. Expression of *EDG*-3 mRNA was detected in MDA-MB-231 cells; however, neither *EDG*-1 nor *EDG*-5 mRNA could be detected. MCF-7 cells expressed *EDG*-3 as well as *EDG*-5, but similarly to MDA-MB-231 cells, did not express *EDG*-1. MCF-7 cells stably overexpressing *EDG*-1 were included as a positive control. To confirm that SPP receptors were constitutively expressed in MCF-7 and MDA-MB-231 cells, we attempted to measure specific binding of [^{32}P]SPP. In both cell lines, total binding of 1 nM [^{32}P]SPP was low and was not significantly reduced by addition of 1000-fold excess of unlabeled SPP, indicating very low expression of specific SPP receptors by these cells. The failure to detect specific SPP binding is not a result of improper binding conditions, since in MCF-7 cells overexpressing EDG-1, significant specific SPP binding could be detected (10.3 $\pm$ 0.6 fmol/10^5 cells).

Effects of SPP Analogs on Chemotaxis. None of the other SPP analogs tested, including the short-chain SPP analog C8-SPP, N-octanoyl ceramide-1-phosphate (C8-Cer-P), cyclic-SPP, or caged-SPP (see below), significantly inhibited motility of MDA-MB-231 cells. Because of the

structural similarities between LPA and SPP, and because they have been reported to bind to the homologous receptors, EDG-2 and EDG-4 **(Goetzl and An 1998)**, we also examined the effect of LPA. Recently it has been suggested that LPA is a low affinity ligand for EDG1 **(Lee et al. 1998)**. However, LPA, even at concentrations as high as 10 μM, had no significant effect on chemotaxis of MDA-MB-231 cells (Fig. 3A in Appendix 2).

EDG-1, -3, and -5, bind SPP with high specificity. Dihydro-SPP, which is similar to SPP but lacks the trans double bond, binds to and signals through all three SPP receptors with similar potency to SPP (Lee et al. 1998; Van Brocklyn et al. 1998; Van Brocklyn et al. 1999). Thus, if inhibition of chemotactic motility is mediated through one or more of these SPP receptors, dihyro-SPP should mimic the effect of SPP. However, 10 μM dihydro-SPP had no significant effect on chemotactic motility of MDA-MB-231 or MCF-7 cells (Fig. 3B in Appendix 2). Collectively, these data suggest that the ability of SPP to inhibit chemotaxis may not be related to binding to EDG cell surface receptors.

Inhibition of Chemotaxis by Caged SPP. The effect of caged SPP photolysis on chemotactic motility of MDA-MB-231 cells was compared to that of exogenously added SPP (Fig. 4 in Appendix 2). Exogenous SPP (10 μM) inhibited chemotactic motility of MDA-MB-231 cells by 50% while caged SPP (5 or 10 μM) had no significant effect on chemotactic motility in non-illuminated cells or in cells illuminated prior to its addition. However, ultraviolet irradiation of caged SPP-loaded cells caused strong inhibition of chemotaxis, whereas ultraviolet irradiation alone had no effect (Fig. 4 in Appendix 2), nor did it alter the inhibitory effect of exogenous SPP. To exclude the possibility that the by-product of caged SPP photolysis, *o*-nitrosoacetophenone, may affect chemotaxis, motility of cells treated with caged cyclic-SPP was measured. Unlike caged SPP, caged cyclic-SPP had no effect on chemotaxis of MDA-MB-231 cells with or without ultraviolet irradiation.

Overexpression of Sphingosine Kinase Decreases Chemotactic Motility of MCF-7 Cells. As an alternative method to increase the level of intracellular SPP, MCF-7 cells were transfected with a murine sphingosine kinase expression vector (Kohama et al. 1998). Transient expression of sphingosine kinase in MCF-7 cells decreased chemotaxis towards FCM (Fig. 5A in Appendix 2). Moreover, although the number of migrating cells was much lower in the absence of

chemoattractant, transient expression of sphingosine kinase also significantly reduced this random motility (Fig. 5A in Appendix 2). Pools of stably transfected cells were used in order to avoid potential phenotypic changes due to selection and propagation of clones derived from single individual cells. Stable expression of sphingosine kinase decreased chemotactic motility of MCF-7 cells by 30% and 46% after 4 h and 24 h, respectively. Because it has been suggested that SPP inhibits melanoma cell motility through an extracellular action by specific binding to cell surface receptors (Yamamura et al. 1997), it was of interest to determine whether MCF-7 cells overexpressing sphingosine kinase, which have increased cellular levels of SPP, can secrete SPP. We have recently developed a sensitive assay for the measurement of SPP (Edsall and Spiegel 1999) that is able to detect as little as 1 pmol SPP. Using this assay, we were unable to detect secretion of SPP into the media by either of the stable pools of sphingosine kinase transfected MCF-7 cells (Table I in Appendix 2), suggesting that SPP is not released by these cells in appreciable amounts. Despite the large increases in [^{32}P]SPP detected in MCF-7 cells overexpressing sphingosine kinase, there was no detectable labeled SPP released into the medium. Based on the sensitivity of these methods, we estimate that the concentration of SPP in the extracellular media is $\leq$0.4 nM, a concentration well below the K_d for binding of SPP to its EDG receptors (Van Brocklyn et al. 1998; Van Brocklyn et al. 1999).

DISCUSSION

SPP, inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 cell line. SPP had no significant effect on the adhesiveness of cells to Matrigel, an extract rich in basement membrane components. Similarly, others have found that although SPP inhibits integrin-dependent motility of mouse melanoma B16 cells, it does not reduce integrin-dependent adhesion to the extracellular matrix (Sadahira et al. 1992). Penetration of the extracellular matrix by metastatic cells requires disruption of local segments of the basement membrane by proteinases, such as matrix metalloproteinase 2 (MMP-2) which has been proposed to play an important role in invasion due to its specificity for basement membrane collagen (Stetler-Stevenson 1990). We

found that only high concentrations of SPP inhibited ConA-induced MMP-2 activation by MDA-MB-231 cells. These results suggest that neither attachment nor MMP-2 activation are critical targets for SPP-dependent inhibition of chemoinvasion. In contrast, one of the critical steps for the invasion of cancer cells, cell motility, was markedly inhibited by SPP. In B16 melanoma cells, SPP strongly inhibited cell motility and phagokinesis at low concentrations (10-100 nM), whereas sphingosine or other related sphingolipids were inactive (Sadahira et al. 1992). Similarly, only SPP inhibited integrin-dependent motility of melanoma cells induced by the extracellular matrix, suggesting that SPP might be acting extracellulary via a cell surface receptor (Sadahira et al. 1994; Yamamura et al. 1997). In contrast, we found that sphingosine, which is readily taken up by cells and phosphorylated to SPP (Zhang et al. 1991), also inhibited motility and invasion of MDA-MB-231 cells, but to a lesser extent than SPP. It was reported that an increased level of FAK expression is highly correlated with invasion potential of human tumor cells (Weiner et al. 1994; Owens et al. 1995), suggesting that FAK may be limiting for cell invasion. FAK has been indicated to play a crucial role in cell migration events (Sankar et al. 1995; Cary et al. 1996; Gilmore and Romer 1996; Brunton et al. 1997). Previously, Guan and co-workers demonstrated that overexpression of FAK, or a KD FAK mutant that was tyrosine phosphorylated and associated with Src, promoted migration of CHO cells on fibronectin (Cary et al. 1996). This effect was dependent on FAK autophosphorylation at Y397 and subsequent binding of Src to this site. We found that overexpression of wild type and KD FAK in MDA-MB-231 cells slightly caused increased chemotactic motility, without reducing the inhibitory effect of SPP. However, expression of autophosphorylation site mutated FAK abolished the effect of SPP on motility. In contrast, wortmannin, a fungal metabolite that directly binds to and inhibits the p110 subunit of PI3-kinase (Arcaro and Wymann 1993), markedly reduced chemotactic motility of these cell lines. Thus, the autophosphorylation site on FAK may play an important role in SPP-mediated signaling leading to decreased cell motility.

Autophosphorylation of FAK at Y397 leads to its association with Src, resulting in activation of both kinases (reviewed in (Hanks and Polte 1997)). The activated FAK/Src complex

phosphorylates several substrates, including tensin, paxillin and p130Cas. FAK phosphorylation and/or binding to paxillin and p130Cas may trigger downstream activation of MAP kinase by the adaptor protein Crk. Src association with FAK may also result in further FAK phosphorylation, forming a docking site for Grb2. However recent results (Cary et al. 1998) demonstrate that p130Cas, but not Grb2, is a mediator of FAK-promoted CHO cell migration. FAK-induced p130Cas phosphorylation occurred when a kinase-defective FAK mutant but not F397 was expressed indicating that Src may be the kinase that mediates phosphorylation of p130Cas (Vuori et al. 1996). This result also suggests that the inability of the Y397F FAK mutant to promote cell migration may be due to inefficient p130Cas phosphorylation and recruitment of signaling molecules. Moreover, it was demonstrated that the catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility (Fincham and Frame 1998). Thus, it was proposed that the Src-FAK-linked activity induces focal adhesion turnover and facilitating cell movement. Further studies are needed to clarify the importance of Src in the mobility inhibiting effect of SPP. However, since Y397 can also bind PI3-kinase, which might be important for motility (Hooshmand-Rad et al. 1997; Keely et al. 1997) , the possibility exists that PI3-kinase is also important for SPP signaling. Moreover, our results indicate that the PI3-kinase pathway can also regulate the motility of MDA-MB-231 cells. However, overexpression of autophosphorylation site mutated FAK did not abrogate the ability of the PI3-kinase inhibitor wortmannin to inhibit motility, suggesting that SPP signaling is clearly independent of the PI3-kinase pathway. Inhibition of cell spreading by expression of the C-terminal domain of FAK is rescued by coexpression of Src or catalytically inactive FAK with concomitant enhancement of tyrosine phosphorylation of paxillin (Richardson et al. 1997). These results suggest that tyrosine phosphorylation of paxillin is a critical step in focal adhesion assembly. In this scenario, FAK may act as a "switchable adaptor" that recruits Src to phosphorylate paxillin, thus promoting focal adhesion assembly.

SPP has been shown to function both intracellularly as a second messenger and at the cell surface through specific SPP receptors to regulate numerous biological processes (Spiegel et al.

1996; Goetzl and An 1998). Previously, based on addition of SPP to cells, many studies have suggested that SPP inhibits cell motility and chemotaxis by binding to a putative cell surface receptor (reviewed in (Yamamura et al. 1997)). SPP has been shown to inhibit cell motility, chemoinvasion, and haptotactic motility (Sadahira et al. 1994) of human B16 melanoma cells in a low concentration range (10 to 100 nM) (Sadahira et al. 1992). These effects appear to be mediated through a cell surface receptor, since SPP immobilized on glass beads, which cannot traverse the cell membrane, mimicked the effects of SPP (Yamamura et al. 1997). In contrast, micromolar concentrations of SPP were necessary to inhibit chemotactic motility of human breast cancer cell lines (MCF-7 and MDA-MB-231) (Wang et al. 1999) and human HT1080 fibrosarcoma cells (Sadahira et al. 1992). It was thus important to determine whether inhibition of breast cancer cell motility by SPP is mediated intracellularly or through a cell surface receptor. Surprisingly, in our study, several lines of evidence indicated that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells through an intracellular action rather than by signaling through cell surface receptors. First, SPP had no effect on motility of MDA-MB-231 cells at concentrations below 1 μM, approximately two orders of magnitude higher than the K_d for binding of SPP to EDG-1, -3, or –5 (Van Brocklyn et al. 1998; Van Brocklyn et al. 1999). Second, although MDA-MB-231 cells express SPP receptor EDG-3 mRNA, and MCF-7 cells express EDG-3 and EDG-5, no specific SPP binding could be detected to either MCF-7 or MDA-MB-231 cells, indicating that either the receptor proteins are not present on the cell surface or that they are expressed at very low levels. Thirdly, dihydro-SPP, which binds to and signals through all three SPP receptors (Van Brocklyn et al. 1998; Van Brocklyn et al. 1999) and Van Brocklyn and Spiegel, unpublished observations), had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. In agreement, it was previously reported that dihydro-SPP did not affect chemotactic motility of human neutrophils (Kawa et al. 1997). Moreover, sphingosine, which is rapidly taken up by cells and converted intracellularly to SPP by sphingosine kinase, also inhibits chemotaxis of MCF-7 and MDA-MB-231 cells (Wang et al. 1999).

Additionally, two independent approaches were used to elevate intracellular SPP bypassing cell surface SPP receptors: treatment of cells with caged SPP, which is taken up by cells and forms SPP intracellularly upon UV irradiation (Qiao et al. 1998); and overexpression of sphingosine kinase, the enzyme which forms SPP within cells. After UV photolysis of intracellular caged SPP, chemotactic motility was inhibited to the same extent as after treatment with exogenous SPP. Overexpression of sphingosine kinase by transfection in MCF-7 cells led to increased intracellular SPP and drastically inhibited chemotactic motility as well as random motility. Although the intracellular levels of SPP were elevated 3 to 4.6 fold in stably transfected cells, no detectable amounts of SPP were released into the medium. Thus, it seems highly unlikely that SPP inhibits motility of breast cancer cells by binding to cell surface receptors. Further studies are needed to clarify the importance of different signaling pathways for controlling motility that are regulated by SPP. Because cell movement is considered as an important step in invasion and metastasis of cancer cells, the finding that endogenous SPP regulates cell migration and chemotactic signaling may have substantial biological ramifications.

CONCLUSIONS

1. Autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to a decrease in cell motility.
2. The inhibitory effect of SPP on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of SPP rather than through cell surface receptors.

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Involvement of Focal Adhesion Kinase in Inhibition of Motility of Human Breast Cancer Cells by Sphingosine 1-Phosphate

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Sphingosine 1-phosphate (SPP), a bioactive sphingolipid metabolite, inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 human breast cancer cell line. As in many other cell types, SPP stimulated proliferation of MDA-MB-231 cells, albeit to a lesser extent. Treatment of MDA-MB-231 cells with SPP had no significant effect on their adhesiveness to Matrigel, and only high concentrations of SPP partially inhibited matrix metalloproteinase-2 activation induced by Con A. However, SPP at a concentration that strongly inhibited invasiveness also markedly reduced chemotactic motility. To investigate the molecular mechanisms by which SPP interferes with cell motility, we examined tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, which are important for organization of focal adhesions and cell motility. SPP rapidly increased tyrosine phosphorylation of FAK and paxillin and of the paxillin-associated protein Crk. Overexpression of FAK and kinase-defective FAK in MDA-MB-231 cells resulted in a slight increase in motility without affecting the inhibitory effect of SPP, whereas expression of FAK with a mutation of the major autophosphorylation site (F397) abolished the inhibitory effect of SPP on cell motility. In contrast, the phosphoinositide 3′-kinase inhibitor, wortmannin, inhibited chemotactic motility in both vector and FAK-F397-transfected cells. Our results suggest that autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to decreased cell motility.

INTRODUCTION

Metastasis is a multifactorial process by which tumor cells escape from the primary tumor, disseminate through blood and lymph vessels, evade host immune defenses, and home to specific target organs where they extravasate and recolonize. Cell movement is considered a crucial step for the invasion of cancer cells [1, 2]. For cellular locomotion to occur, the cell must be able to protrude, form and break adhesion sites between the cell and the substratum, and move the cell mass. The assembly of focal adhesions, structures that link actin filaments and integrin-associated points of attachment with the extracellular matrix [3], and the concomitant recruitment of actin stress fibers and other cytoskeletal proteins are important for cell migration [4, 5]. Increased tyrosine phosphorylation of several proteins which are associated with focal adhesions has been implicated in cell migration and invasion [2, 5]. Among them, focal adhesion kinase (FAK),[2] is a widely expressed and highly conserved non-receptor protein tyrosine kinase that has been implicated in organization of focal adhesions [6], formation of actin stress fibers [7, 8] and regulation of motility [9–11] and invasion [12, 13]. FAK, in turn, phosphorylates and/or associates with other cytoskeletal components, including Src, the Crk-associated substrate p130Cas (Cas), paxillin, and tensin [5, 14], that will either lead to activation of signaling pathways or establish contacts with talin, vinculin, α-actinin, and the actin polymers, essential components of stress fiber regulation.

Recent studies indicate that the sphingolipid metabolites ceramide, sphingosine, and sphingosine 1-phosphate (SPP) can enhance tyrosine phosphorylation of FAK, leading to stress fiber formation and focal contact assembly in Swiss 3T3 cells [7, 15]. Moreover, in human arterial smooth muscle cells, SPP generated in response to PDGF interfered with the dynamics of PDGF-stimulated actin filament disassembly and assembly, resulting in a marked inhibition of cell spreading, extension of the leading lamellae, and chemotaxis toward PDGF [16]. These results suggest that endogenous SPP may play an important role in regulating cell

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[2] Abbreviations used: IMEM, Richter's improved minimal essential medium; FBS, fetal bovine serum; BSA, bovine serum albumin; FAK, focal adhesion kinase; KD, kinase defective; FACS, flow cytometric cell sorting; mAb, monoclonal antibody; MMP-2, matrix metalloproteinase-2; Con A, concanavalin A; SPP, sphingosine 1-phosphate; Sph, sphingosine; C2-Cer, *N*-acetylsphingosine; C6-Cer, *N*-hexanoylsphingosine; PI3-kinase, phosphatidylinositide 3′-kinase.

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migration and chemotactic signaling. In contrast, other studies suggest that SPP acts through a cell surface receptor to inhibit motility [17] and induce cell rounding [18]. At very low concentrations (10 nM), SPP, but not sphingosine, effectively inhibited chemotactic motility of several tumor cell lines, including mouse and human melanoma and human osteosarcoma cells, whereas the motility of endothelial cells was not affected [19, 17]. However, the mechanism by which SPP inhibits motility is still not understood. In this study, we examined the effects of SPP on invasiveness and motility of the highly invasive MDA-MB-231 human breast cancer cell line and also examined the involvement of FAK. Our experiments suggest that autophosphorylation of FAK on Y397 may play an important role in SPP signaling leading to a decrease in cell motility.

MATERIALS AND METHODS

Materials. The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the Cell Culture Core Resource, Lombardi Cancer Center (Washington, DC). Media and supplements were from Biofluids, Inc. (Bedford, MA). Collagen type IV was purchased from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). [*methyl*-^{3}H]Thymidine (55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Sphingosine and SPP were obtained from Biomol Research Laboratory, Inc. (Plymouth Meeting, PA). Sphingosine, *N*-hexanoyl sphingosine (C6-Cer), and *N*-acetyl sphingosine (C2-Cer) were from Matreya (Pleasant Gap, PA). Gelatin was from NOVEX (Encinitas, CA). Anti-FAK mAb was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphotyrosine (PY20), anti-paxillin, anti-Crk, and anti-β1 integrin mAbs were obtained from Transduction Laboratories (Lexington, KY). Protein A/G-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Bovine serum albumin, L-ascorbic acid, and Coomassie Blue G-250 were from Sigma Chemical Co. (St. Louis, MO). Anti-KT3 antibody was from Babco (Richmond, CA). Wortmannin was from Alexis Biochemicals (San Diego, CA). G418 was from Mediatech (Herndon, VA). ECL was from Pierce (Rockford, IL).

Cell culture. Cells were maintained in Richter's improved minimal essential medium (IMEM), supplemented with 5% fetal bovine serum (FBS). Unless indicated, cells were seeded at 1.8×10^4 cells/cm^2. Twenty-four hours prior to experiments, cells were changed to serum-free IMEM supplemented with 2 mM Hepes; 1% (v/v) nonessential amino acids; 1% (v/v) trace elements; 0.4% (v/v) insulin, transferrin, and selenium; 0.2% (w/v) fibronectin; and 1% (v/v) vitamins and treated with different reagents as indicated.

Chemoinvasion assay. Boyden chamber chemoinvasion assays were carried out essentially as described previously using two sizes of chambers (growth area 18 mm^2 or 50 mm^2) [20]. Briefly, polycarbonate filters (13-mm diameter, 12-μm pore size) were coated with Matrigel (25 μg), a murine tumor extract rich in basement membrane components (particularly laminin, collagen type IV, and heparan sulfate proteoglycan) for 1 h at 37°C, rinsed once with PBS, and then placed into the lower chamber. Fibroblast conditioned medium, obtained by incubating confluent NIH 3T3 cells for 24 h with IMEM supplemented with L-ascorbic acid (50 μg/ml) or fetal bovine serum were placed in the lower chamber as chemoattractants. Cells were harvested by trypsinization, washed twice with IMEM containing 0.1% bovine serum albumin (IMEM/BSA), and added to the upper chamber at 0.75×10^5 (for small chamber) or 3×10^5 cells per well (for large chamber) and treated as indicated. The chambers were incubated in a humidified incubator at 37°C in 5% CO_2/95% air for 4 h. The cells which traversed the Matrigel and spread on the lower surface of the filter were fixed in methanol for 8 min and stained with crystal violet. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells per membrane was enumerated using light microscopy at 10× magnification. Each data point is the average number of cells in four random fields, each counted twice. Each determination represents the average of three individual wells, and error bars represent SD. In some experiments, cells were pretreated with the indicated sphingolipid metabolites for 24 h before the chemoinvasion assay.

[^{3}H]Thymidine incorporation. Cells were cultured in serum-free IMEM in the presence of different mitogens for 18 h and then pulsed with [^{3}H]thymidine (1 μCi/ml) for 6 h. The incorporation of radioactivity into trichloroacetic acid-insoluble material was then measured [21]. Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

Flow cytometric analysis. Cells were treated without or with SPP for 24 h and trypsinized to obtain single-cell suspensions. After centrifugation, cells (1×10^6) were resuspended in 100 μl citrate buffer (250 mM sucrose, 40 mM trisodium citrate, and 0.05% (v/v) DMSO, pH 7.6). Cell nuclei were stained with propidium iodide and analysis of cell cycle distribution was performed with a FACStar^{plus} flow cytofluorometer (Becton–Dickinson, San Jose, CA).

Adhesion assay. Matrigel (2.5 μg) was incubated in each well of a 96-well culture plate for 60 min at 37°C. The plates were then incubated with 3% BSA in PBS for 30 min to block nonspecific binding sites followed by three washes with PBS. After treatment with the indicated amount of SPP for 6 or 24 h, cells were harvested by scraping in 10 mM EDTA in PBS, washed twice with IMEM/BSA, resuspended at 5×10^5 cells/ml in IMEM/BSA containing the indicated amounts of SPP, and incubated for 1 h at 37°C. Cell suspensions (100 μl) were then added to each well and incubated at 37°C for 1 h. Nonadherent cells were removed and the attached cells fixed with 70% ethanol for 20 min and stained with crystal violet (5 mg/ml in 20% methanol) for 10 min. Wells were gently rinsed three times with water and allowed to dry. Incorporated dye was dissolved in 100 μl/well of 0.1 M sodium citrate in 50% ethanol (pH 4.2) and the absorbance measured at 540 nm.

Zymography. Cells in 24-cluster plates (5×10^4 cells/cm^2) were washed with IMEM and changed to serum-free conditioned medium (serum-free medium conditioned by MMP-2-transfected MCF-7 cells as the source of latent MMP-2) as previously described [22]. Cells were treated with Con A in the absence or presence of SPP. The conditioned medium was collected after 24 h and MMP-2 activation analyzed by zymography on 10% SDS–PAGE gels copolymerized with 2 mg/ml gelatin (NOVEX, Encinitas, CA). The activity of the latent 72-kDa species of MMP-2 can be visualized after exposure of the active site by SDS while MMP-2 activation is indicated zymographically by the size reduction to the intermediate and/or mature enzyme forms of 62 and 59 kDa, respectively. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h at room temperature to remove SDS. Zymograms were developed by incubation overnight in collagenase buffer (0.2 M NaCl, 5 mM $CaCl_2$, 1% (v/v) Triton X-100, and 0.02% NaN_3 (w/v) in 50 mM Tris–HCl, pH 7.4). Zymograms were stained with 1% (w/v) Coomassie Blue G-250 dissolved in 30% methanol containing 10% glacial acetic acid. After destaining, gelatinolytic activities were visualized as clear bands against a dark background of stained gelatin.

Chemotaxis assay. Chemotaxis was measured as described for the chemoinvasion assay with the exception that filter surfaces were coated with 5 μg of collagen IV instead of Matrigel [20]. Collagen IV coatings promote uniform attachment to and migration across the filter, without formation of a barrier. Since the cells can migrate without degrading the collagen, this assay measures chemotaxis rather than chemoinvasion.

Measurement of uptake and metabolism of SPP. Cells were incubated for 30 min at 37°C in the presence of 10 μM [^{32}P]SPP (64,000 cpm/nmol), washed with medium containing 0.4 mg/ml fatty acid-

free BSA, and then incubated at 37°C for the indicated time [23]. Cells were trypsinized and cellular lipids were extracted as described [24]. Extracts were applied to silica gel 60 G plates which were developed with chloroform/methanol/25 mM NaH_2PO_4 (60/35/8, v/v) and then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA). Radioactive bands were quantified using a Storm densitometer with the ImageQuant program (Molecular Dynamics). Sphingosine production was measured as described [25].

Immunoprecipitation. Cells were treated with the indicated sphingolipids, washed twice with PBS, and lysed at 4°C in 500 μl of lysis buffer consisting of 50 mM Hepes, pH 7.4/1% (v/v) Triton X-100/150 mM NaCl/1 mM EDTA/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride/50 mM NaF/4 mM sodium pyrophosphate/2 mM Na_3VO_4/leupeptin and aprotinin (2.5 μg/ml each). After centrifugation at 14,500*g*, 350- to 500-μg aliquots of supernatant proteins were incubated with 3–4 μg mAbs directed against FAK, paxillin, or Crk for 4 h at 4°C, followed by the addition of 20 μl of protein A/G–agarose. After incubating overnight at 4°C, the immune complexes bound to agarose were recovered by centrifugation, washed twice with lysis buffer, extracted for 10 min at 95°C in 2× SDS–PAGE sample buffer (200 mM Tris, 2 mM EDTA, 6% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, pH 6.8), and then analyzed by SDS–PAGE.

Western blotting. After SDS–PAGE, proteins were transferred to PVDF membranes, blocked with 3% (w/v) BSA in PBS, and incubated with anti-Tyr(P) mAb (PY20H, 1:2500) or mAb directed against FAK, paxillin, or Crk as indicated. Immunoreactive bands were detected by ECL using horseradish peroxidase-conjugated anti-mouse IgG. In some cases, the immunoblots were stripped and reprobed with antibodies according to the manufacturer's recommendations (ECL; Amersham Corp., Arlington Heights, IL).

Transfection. The cDNAs encoding FAK and its mutants contain an epitope tag derived from the final 11 COOH-terminal residues of SV40 large T antigen (KPPTPPPEPET). KD is a kinase-defective FAK mutant with a lysine 454 to arginine mutation in the ATP binding site of the kinase domain. F397 contains a mutation at the autophosphorylation site (Tyr 397 to Phe mutation). Epitope-tagged FAK and its mutants were cloned into vector pCDM8 to generate the expression plasmids pCDM8-FAK, pCDM8-KD, and pCDM8-F397, as described previously [9], and were kindly provided by Dr. J. L. Guan. MDA-MB-231 cells were cotransfected with 20 μg of the expression plasmids or vector together with 2 μg of pEGFP-C3 using the calcium phosphate precipitation method [26]. pEGFP-C3 encodes a green fluorescent protein and contains a neomycin-resistance cassette. After 48 h, transfected cells expressing high levels of GFP were selected and isolated by FACS analysis. Neomycin-resistant cells were selected in growth media containing 0.9 mg/ml G418 for 20 days and maintained in the same media. Transfected cells were then screened for exogenous FAK expression by Western blotting using the mAb KT3.

RESULTS

SPP inhibits chemoinvasiveness of MDA-MB-231 cells. To examine whether sphingolipid metabolites affect invasion, MDA-MB-231 cells were pretreated with SPP, sphingosine, cell-permeable ceramide analogs (C2-Cer and C6-Cer), or sphingomyelinase (which cleaves plasma membrane sphingomyelin to form ceramides) for 24 h, and then chemoinvasion toward fibroblast conditioned medium was measured. As can be seen in Fig. 1A, SPP, but not sphingomyelinase treatment or ceramide analogs, inhibits invasion. A significant inhibitory effect was found at 5 μM SPP and a maximal effect was observed at 10–20 μM. Sphingosine, at 20 μM, has a smaller inhibitory effect. Similarly, SPP inhibits chemoinvasion toward FBS. No cytotoxic effects were detected for any of these sphingolipids up to concentrations of 5 μM, but ceramide analogs were cytotoxic at concentrations ≥10 μM. In contrast, sphingosine and SPP were not cytotoxic up to 20 and 50 μM, respectively. Similar results were obtained when cells were treated with sphingosine or SPP for only 4 h during the chemoinvasion toward fibroblast conditioned medium and FBS (Fig. 1B). However, the inhibitory effects required higher concentrations when incubated with the cells for 4 h rather than 24 h.

Since SPP inhibits chemoinvasiveness more potently than sphingosine, and ceramide is completely inactive, it seems most likely that SPP itself is the active molecule. To examine this issue further, we have studied the cellular uptake and metabolism of SPP. SPP was rapidly taken up by MDA-MB-231 cells as the intact molecule, and maximum uptake occurred within 5 min. This was followed by a slower phase of metabolism to sphingosine, whose level peaked after 60 min, most probably catalyzed by a specific phosphatase which has recently been identified in *Saccharomyces cerevisiae* [27]. Some of the effects of exogenously added SPP might be due to binding to a specific cell surface receptor. Recently, we have identified endothelial cell differentiation gene-1 (*edg-1*) as the G-protein-coupled receptor for SPP [28, 23]. However, we could only detect extremely low levels of specific SPP binding (9 fmol/million cells) and MDA-MB-231 cells have no detectable *edg-1* mRNA as determined by RT-PCR and Northern analysis (data not shown).

Effects of sphingosine 1-phosphate on proliferation of MDA-MB-231 cells. SPP is a potent mitogen for many different cell types [29] and also induced a moderate increase in DNA synthesis of MDA-MB-231 (Fig. 2A) and MCF-7 cells (data not shown) as measured by [^{3}H]thymidine incorporation. Consistent with these results, flow cytometric analysis of cell cycle distribution revealed that optimally mitogenic concentrations of SPP (10 μM) induced a slight increase in the proportion of MDA-MB-231 cells in S phase, with a concomitant decrease in the proportion of cells in G_0–G_1 (Fig. 2B). It should be pointed out that despite the increase in S phase, no changes could be detected in cell numbers. In contrast, similar concentrations of SPP induced a significant increase in cell numbers of quiescent Swiss 3T3 fibroblasts [21].

SPP inhibits MMP-2 activation and has no effect on adhesion of MDA-MB-231 cells to Matrigel. There are three major steps of invasion: adhesion, extracellular matrix digestion, and cell movement. To determine whether the effect of SPP on invasion was due to altered adhesion, cells were pretreated with SPP or anti-β1 integrin antibody (20 μg/ml) for 24 h, and adher-

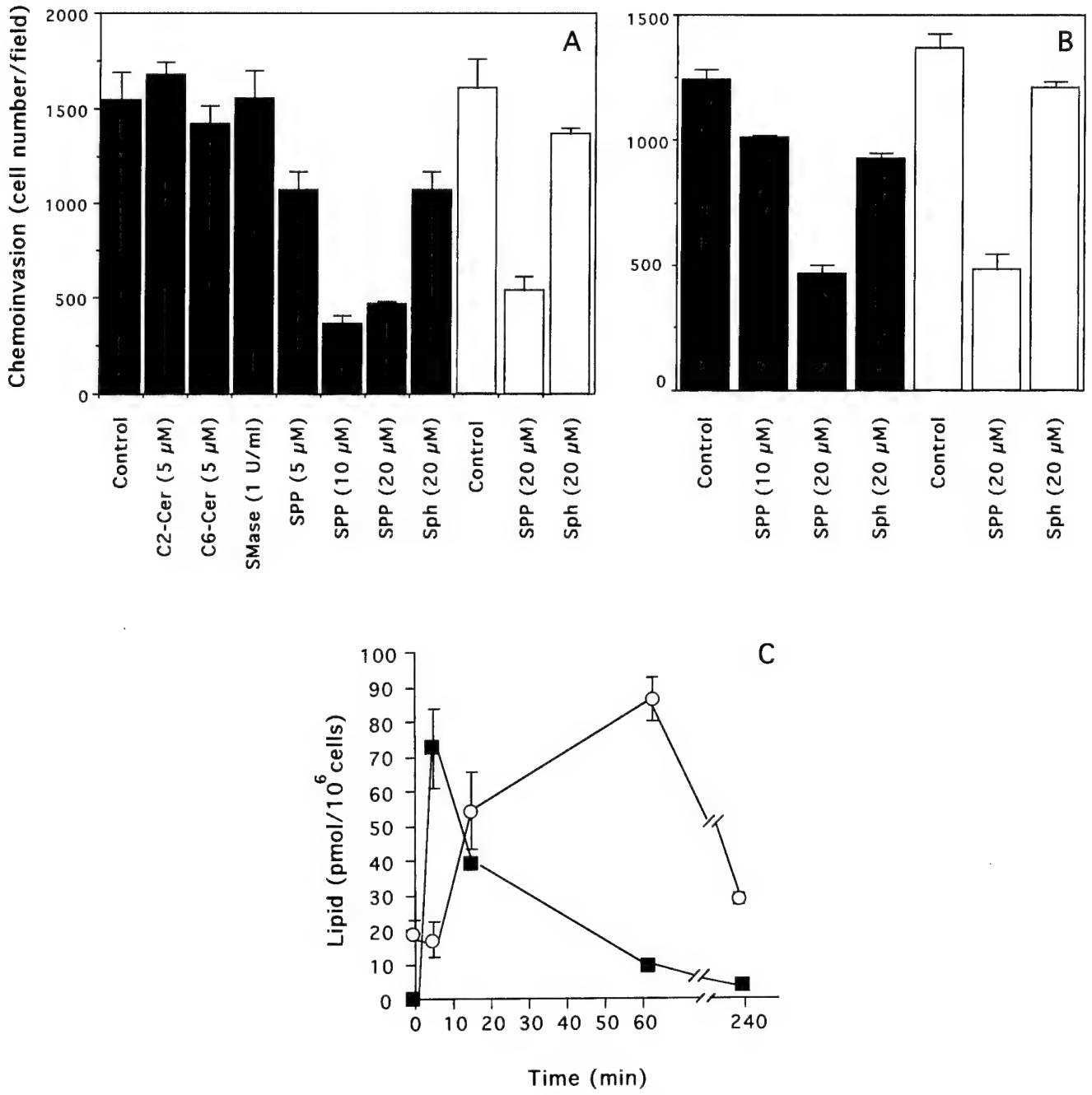

FIG. 1. SPP reduces chemoinvasion of MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with vehicle (Control), 5 μM *N*-acetylsphingosine (C2-Cer), 5 μM *N*-hexanoylsphingosine (C6-Cer), 1 U/ml sphingomyelinase (SMase) from *Streptomyces* sp., or the indicated concentrations of SPP and sphingosine (Sph) for 24 h; harvested with trypsin; counted; and allowed to migrate through polycarbonate filters coated with 25 μg of Matrigel for 4 h in the presence of the indicated agents. Fibroblast conditioned medium (filled bars) or FBS (open bars) was added to the lower chamber as chemoattractant, and chemoinvasion was measured as described under Materials and Methods. The basal chemoinvasion in the absence of chemoattractants was 60 cells/field. The results are from a representative experiment repeated four times. All treatments except Cer-2, Cer-6, and SMase were significantly different from control as determined by Student's *t* test ($P < 0.05$). (B) MDA-MB-231 cells were treated with vehicle (Control) or the indicated concentrations of SPP and Sph during migration for 4 h through polycarbonate filters coated with 25 μg of Matrigel. Fibroblast conditioned medium (filled bars) or FBS (open bars) was added to the lower chamber as chemoattractants. All treatments were significantly different from control as determined by Student's *t* test ($P < 0.05$). (C) Kinetics of uptake and metabolism of SPP. MDA-MB-231 cells were incubated at 37°C with 10 μM SPP for the indicated times. SPP (filled square) and sphingosine (open circle) were analyzed as described under Materials and Methods.

ence to Matrigel was determined (Fig. 3A). Treatment of MDA-MB-231 cells with SPP at concentrations that strongly inhibited invasiveness (5–10 μM) had no significant effects on the adhesiveness of cells to Matrigel. As expected, pretreatment of cells with anti-integrin β1 antibody strongly inhibited adhesion to Matrigel

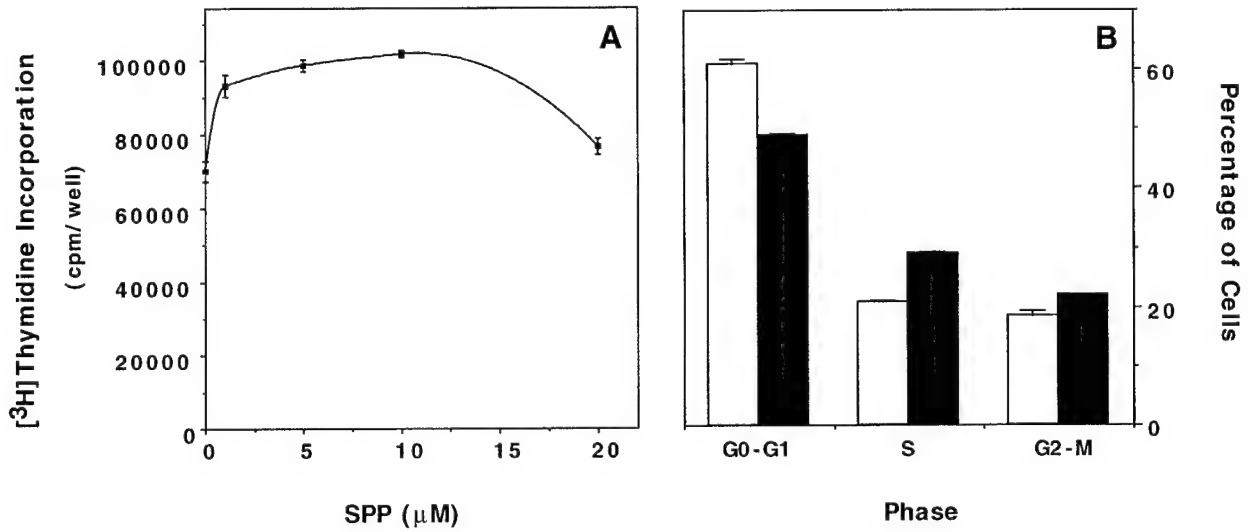

FIG. 2. Effect of SPP on growth of MDA-MB-231 cells. (A) [^{3}H]Thymidine incorporation. MDA-MB-231 cells (1.8×10^4 cells/cm^2) were incubated in chemically defined medium in the absence or presence of various concentrations of SPP for 24 h and [^{3}H]thymidine incorporation was measured as described under Materials and Methods. Data are the means ± SD of triplicate determinations. Similar results were obtained in at least five additional experiments. (B) Flow cytometric analysis of cell cycle distribution. Cells were incubated in chemically defined medium in the absence (open bars) or presence (filled bars) of 10 μM SPP. After 24 h, cells were analyzed by FACS as described under Materials and Methods. Results (mean ± SD; $n = 3$) are expressed as percentage of cells in each of the cell cycle phases. The changes in the percentage of cells in G_o–G_1 and S phases were significantly different from control as determined by Student's t test ($P < 0.05$).

(Fig. 3A), while treatment of cells with an unrelated antibody had no effect.

To penetrate the extracellular matrix, metastatic cells disrupt local segments of the basement membrane with proteinases, such as MMP-2 [30]. In breast adenocarcinomas, MMP-2 is secreted by the reactive stroma and appears to be sequestered by the carcinoma cells [31]. Certain breast carcinoma cells, including MDA-MB-231, are able to activate MMP-2 when treated with Con A [22, 32]. MMP-2 is secreted as a proenzyme (72 kDa) whose proteolytic activity is triggered by truncation and conformational rearrangement at the cell surface [33]. Due to the importance of activation of MMP-2 in matrix degradation in breast cancer [34], the effect of SPP on MMP-2 activation by MDA-MB-231 cells was examined. In agreement with previous studies [22, 32], Con A induced activation of MMP-2 (Fig. 3B, lane 2). Whereas SPP alone did not significantly affect MMP-2 activation (lane 4), it inhibited Con A-induced activation of MMP-2 only at high concentrations (lane 3). Although the concentration of SPP required for the inhibitory effects in the zymograph assay was higher than that required to inhibit chemoinvasiveness, the zymographic assay is relatively less sensitive and more cells are required (2.7-fold higher density of cells). Therefore, the relative amount of SPP added per cell may be comparable in both assays. Our results indicate that MMP-2 activation does not appear to be a major target of SPP in inhibition of chemoinvasion by these cells.

SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells. One of the critical steps for the invasion of cancer cells is cell movement [1, 2]. Cell motility can be random (chemokinesis), directed toward concentration gradients of various attractants, including growth factors (chemotaxis), or directed toward extracellular matrix-bound components (haptotaxis) [35]. Previously, low concentrations of SPP have been shown to affect motility of various types of cells [19, 17]. Thus, we examined the effects of SPP on chemoattractant-induced cell motility of MDA-MB-231 (Fig. 4A) and MCF-7 cells (Fig. 4B). As previously reported, MDA-MB-231 cells are much more motile than the MCF-7 cells (cell number per field was 1720 ± 80 for control MDA-MB-231 and 520 ± 75 for MCF-7 cells). SPP markedly inhibited chemotaxis at 5–10 μM in both cell lines. Similar to the results of the chemoinvasion assays, SPP was more potent than sphingosine at inhibiting motility of MDA-MB-231 cells (Fig. 4A). Cells treated with SPP for 4 h in the chamber without pretreatment also showed decreased motility compared with untreated cells (Fig. 4C). However, in this case, maximal inhibitory effects required higher concentrations of SPP.

SPP increases tyrosine phosphorylation of focal adhesion kinase. Little is yet known of the molecular mechanisms by which SPP regulates cell motility. Tyrosine phosphorylation and activation of FAK have been implicated in the regulation of cell motility [36]. Moreover, we have recently shown that SPP stimulates

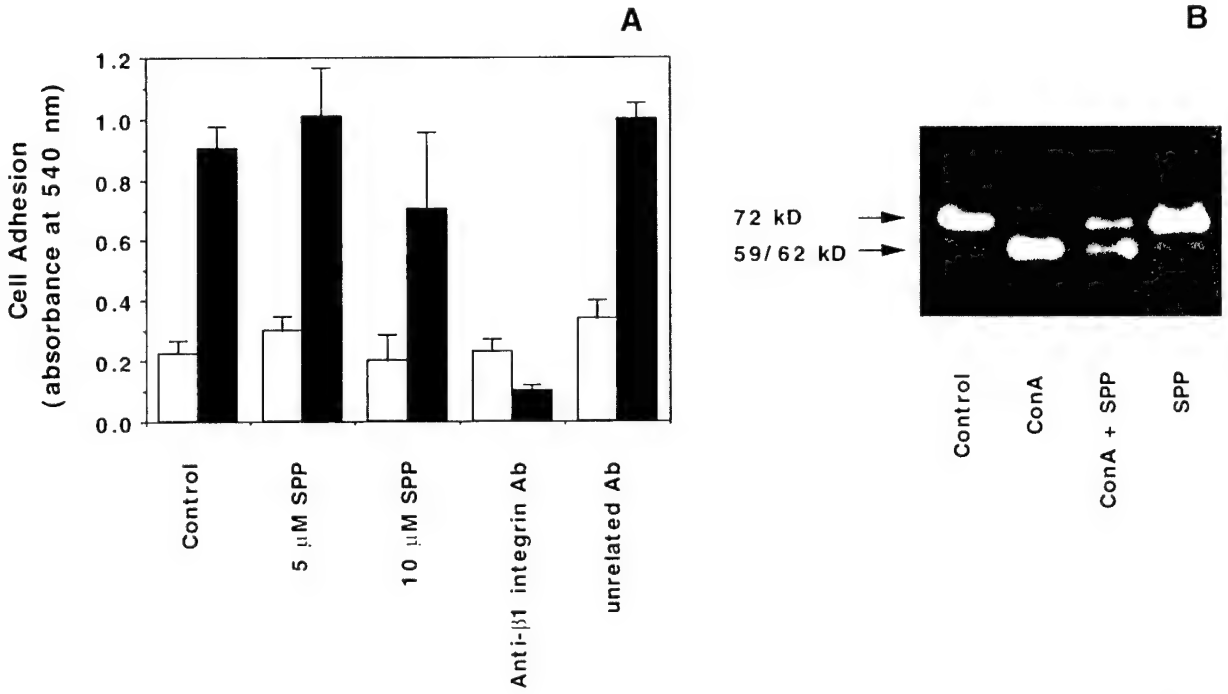

FIG. 3. Effects of SPP on cell adhesion to Matrigel and Con A-induced activation of metalloproteinase 2. (A) MDA-MB-231 cells were treated with vehicle, the indicated concentrations of SPP for 24 h, anti-β1 integrin, or unrelated antibody (20 μg/ml) for 1 h, and attachment of cells to plastic (open bars) and to plates coated with Matrigel (filled bars) was determined as described under Materials and Methods. (B) MDA-MB-231 cells (5×10^4 cells/cm^2) were treated with Con A in the absence or presence of SPP (40 μM) in medium containing latent MMP-2. After 24 h, MMP-2 in the medium was analyzed by zymography as described under Materials and Methods. Latent (72 kDa) and mature (62/59 kDa) forms of MMP-2 are indicated.

tyrosine phosphorylation of FAK in quiescent Swiss 3T3 fibroblasts [15], whereas no effect on FAK phosphorylation could be detected in human arterial smooth muscle cells whose chemotactic mobility was markedly inhibited by SPP [16]. To examine the effects of SPP on FAK phosphorylation in MDA-MB-231 cells, lysates were immunoprecipitated with anti-FAK mAb and analyzed by SDS–PAGE followed by immunoblotting with anti-phosphotyrosine antibody. SPP (10 μM) induced rapid tyrosine phosphorylation of FAK, reaching maximal levels within 5 min and declining thereafter (Fig. 5A). Treatment of cells with 10 μM sphingosine also induced tyrosine phosphorylation of FAK (Fig. 5B), whereas the cell-permeable ceramide analog C6-Cer had almost no effect (Fig. 5C).

SPP increases tyrosine phosphorylation of paxillin and Crk. In many cell types, FAK binds and phosphorylates other cytoskeletal components, particularly paxillin and tensin [5], that subsequently will establish contacts with other essential components of the cytoskeleton. SPP (10 μM) induced a time-dependent increase of paxillin phosphorylation with maximal effect at 5 min (Fig. 6A), declining after 15 min and returning to basal levels after 60 min. Sphingosine (20 μM) also induced phosphorylation of paxillin within 5 min of treatment (Fig. 6B).

It has been suggested that phosphorylated paxillin may serve as a molecular adaptor, responsible for the recruitment of structural and signaling molecules to focal adhesions. The adaptor protein Crk has been shown to bind to tyrosine-phosphorylated paxillin through its Src-homology 2 domain [37], which may trigger downstream activation of MAP kinase. Similar to previous reports in other cell types [38, 39], SPP markedly increased tyrosine phosphorylation of Crk. Within 5 min of treatment with SPP, there was a significant increase in tyrosine phosphorylation of the slower migrating Crk band without affecting that of the faster migrating protein (Fig. 6C). Sphingosine (20 μM) induced Crk phosphorylation to a lesser extent than SPP (10 μM). The two closely migrating proteins correspond to Crk as determined with Crk mAb.

The major autophosphorylation site of FAK, Y397, is important for inhibition of motility of MDA-MB-231 cells by SPP. Recent studies demonstrate the importance of the major autophosphorylation site Y397 of FAK for CHO cell migration on fibronectin [9]. Overexpression of FAK or a kinase-defective FAK mutant in CHO cells resulted in increased migration, whereas CHO cells expressing the FAK point mutant F397, which did not bind Src, demonstrated a basal level of cell migration. To examine the role of FAK in MDA-MB-231 cells, we utilized these FAK constructs. After transfection and FACS isolation, cells expressing

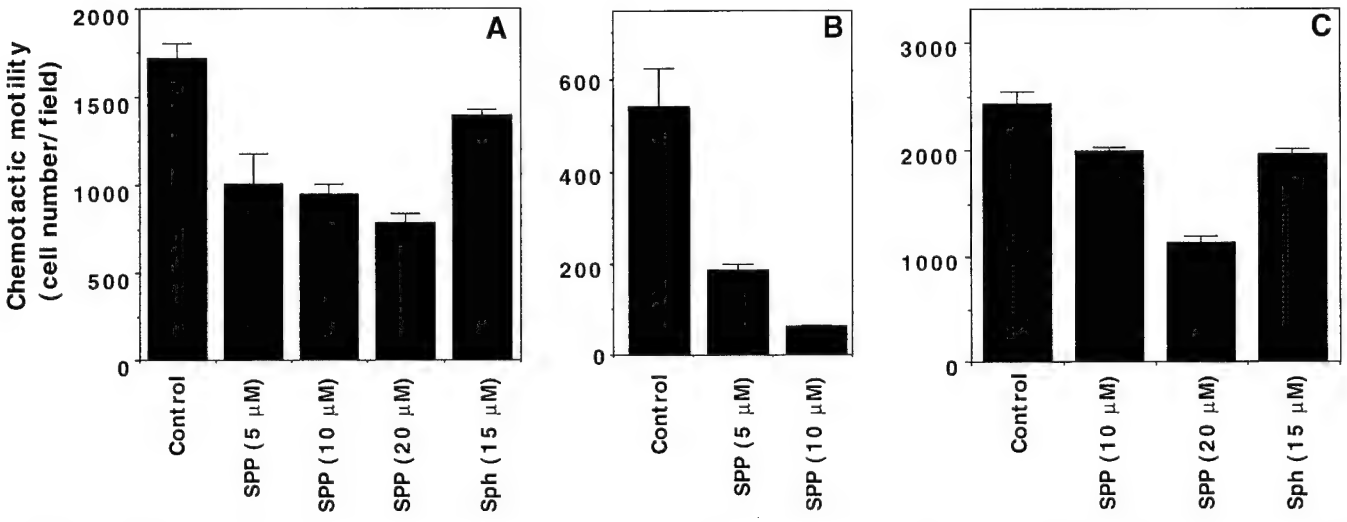

FIG. 4. SPP markedly inhibits chemotaxis of MDA-MB-231 and MCF-7 cells. MDA-MB-231 (A) or MCF-7 (B) cells were pretreated with the indicated concentrations of SPP or sphingosine (Sph) for 24 h, harvested, counted, and allowed to migrate for 4 h through polycarbonate filters coated with 5 μg of collagen IV. Chemotaxis was measured as described under Materials and Methods. The basal chemotactic motility in the absence of chemoattractants was 120 ± 14 and 40 ± 5 cells/field in MDA-MB-231 and MCF-7 cells, respectively. (C) MDA-MB-231 cells were treated with vehicle (Control) or the indicated concentrations of SPP and Sph during chemotaxis for 4 h. All treatments were significantly different from control as determined by Student's *t* test ($P < 0.05$).

epitope-tagged WT, kinase-defective, or F397 FAK were screened by immunostaining with anti-KT3 antibody. A protein (125 kDa) which was recognized by the KT3 antibody was detected in all three FAK-transfected cell lines but not in control cells transfected with vector alone (Fig. 7A). Total FAK expression was also increased after transfection as detected by Western blotting with anti-FAK antibody (Fig. 7A).

To determine the effects of FAK phosphorylation on cell migration, chemotactic motility of these cell lines was analyzed by Boyden chamber assays. In agreement with previous studies [9], the WT and KD cell lines exhibited slightly increased levels of migration compared to vector-transfected cells, whereas expression of F397 resulted in decreased motility (Fig. 7B). These results suggest that, similar to other cell types, autophosphorylation of Y397 of FAK is necessary for FAK-dependent motility of MDA-MB-231 cells.

Because SPP increased tyrosine phosphorylation of FAK and its associated substrates, it was of interest to

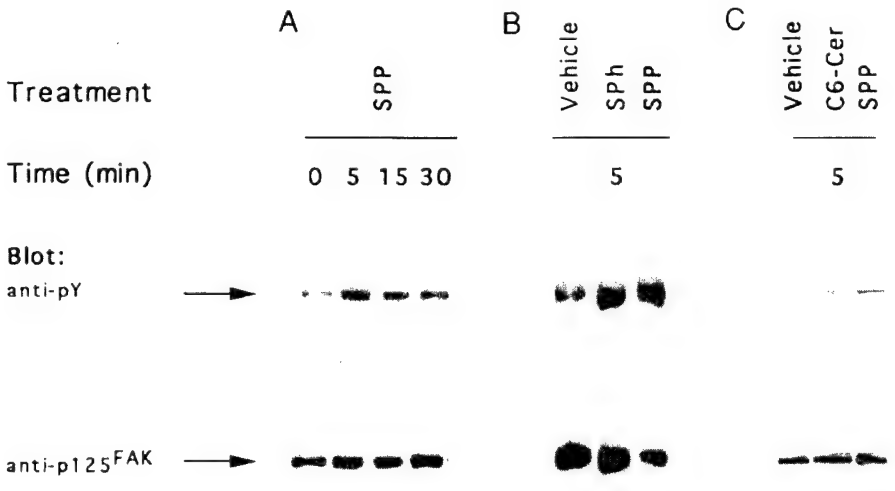

FIG. 5. SPP induces FAK tyrosine phosphorylation. In A, MDA-MB-231 cells were cultured in FBS/IMEM, washed, and treated in chemically defined medium with 10 μM SPP for the indicated times. In B and C, cells were treated for 5 min with the sphingolipid metabolites: SPP (10 μM), Sph (20 μM), C6-Cer (5 μM). Cell lysates were immunoprecipitated with anti-p125^{FAK} mAb and analyzed by immunoblotting with anti-phosphotyrosine (top) or anti-p125^{FAK} antibody (bottom). The data are representative results from at least three independent experiments.

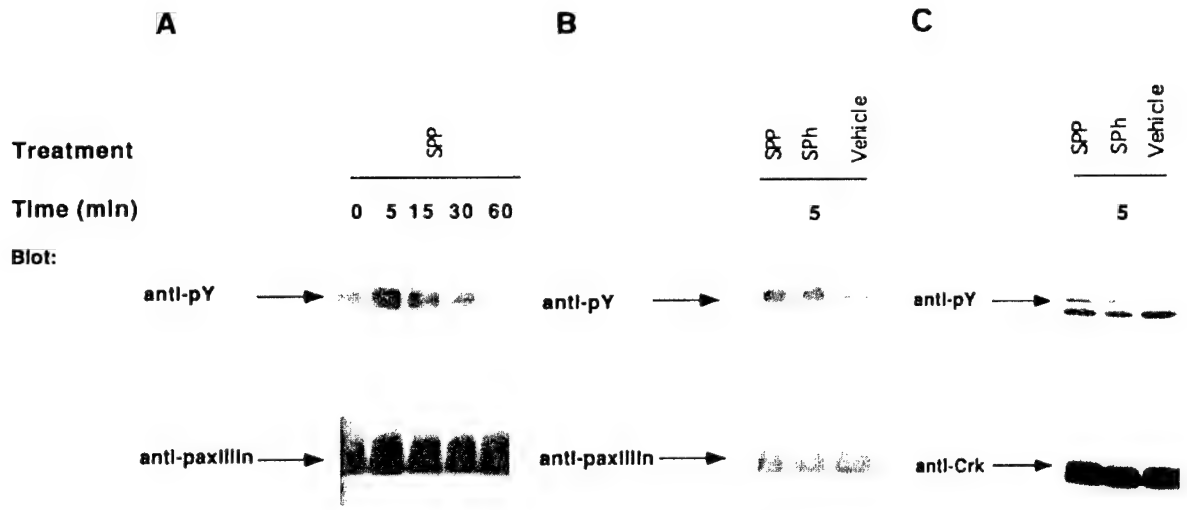

FIG. 6. SPP stimulates tyrosine phosphorylation of paxillin and Crk. MDA-MB-231 cells were treated (A) with SPP (10 μM) for the indicated times or (B) with Sph (20 μM) or SPP (10 μM) for 5 min. Cell lysates were immunoprecipitated with anti-paxillin mAb and the precipitates resolved on SDS–PAGE gels, transblotted, and analyzed by Western blotting with anti-Tyr(P) antibody (top) or anti-paxillin antibody (bottom). (C) MDA-MB-231 cells were treated with SPP (10 μM) or Sph (20 μM) for 5 min. Lysates were immunoprecipitated with anti-Crk mAb and analyzed by Western blotting with anti-Tyr(P) antibody (top) or anti-Crk antibody (bottom).

examine the involvement of FAK in SPP signaling leading to decreased cell motility. SPP markedly inhibited motility of cells overexpressing WT and KD FAK but not of cells expressing autophosphorylation-site-mutated FAK (Fig. 7B). Moreover, the effect of SPP on WT cells was time-dependent (Fig. 7C). Inhibition of motility was proportional to the length of time that the cells were exposed to SPP. However, SPP did not have any effect on motility of F397 cells at the time period examined. It is possible that overexpression of F397 might cause MDA-MB-231 to become unresponsive to any motility-inhibiting drugs. Inhibition of PI3-kinase by wortmannin [40] has been shown to inhibit motility induced by PDGF [41], cdc42, and Rac1 [42]. As shown in Fig. 7D, wortmannin strongly inhibited chemotactic motility in both vector-transfected and F397 cells. These results indicate that the motility of these cells is not generally compromised by overexpression of autophosphorylation-site-mutated FAK and suggest that Y397 is essential for SPP-induced inhibition of motility which is independent of the PI3-kinase pathway.

DISCUSSION

SPP inhibits chemoinvasiveness of the aggressive, estrogen-independent MDA-MB-231 cell line. Three critical steps have been identified in the process of invasion of cancer cells: attachment of tumor cells to the basement membrane, extracellular matrix digestion, and subsequent cellular movements [30]. SPP had no significant effect on the adhesiveness of cells to Matrigel, an extract rich in basement membrane components. Similarly, others have found that although SPP inhibits integrin-dependent motility of mouse melanoma B16 cells, it does not reduce integrin-dependent adhesion to the extracellular matrix [19]. Penetration of the extracellular matrix by metastatic cells requires disruption of local segments of the basement membrane by proteinases, such as matrix metalloproteinase 2, which has been proposed to play an important role in invasion due to its specificity for basement membrane collagen [33]. We found that only high concentrations of SPP inhibited Con A-induced MMP-2 activation by MDA-MB-231 cells. These results suggest that neither attachment nor MMP-2 activation is a critical target for SPP-dependent inhibition of chemoinvasion. In contrast, one of the critical steps in the invasion of cancer cells, cell motility, was markedly inhibited by SPP. In B16 melanoma cells, SPP strongly inhibited cell motility and phagokinesis at low concentrations (10–100 nM), whereas sphingosine or other related sphingolipids were inactive [19]. Similarly, only SPP inhibited integrin-dependent motility of melanoma cells induced by the extracellular matrix, suggesting that SPP might be acting extracellularly via a cell surface receptor [43, 17]. In contrast, we found that sphingosine, which is readily taken up by cells and phosphorylated to SPP [21], also inhibited motility and invasion of MDA-MB-231 cells, but to a lesser extent than SPP. Moreover, the inhibitory effects of SPP were observed only at 5–20 μM, concentrations well above the K_m of Edg-1, the receptor for SPP (8 nM). In addition, there is no specific binding of SPP to MDA-MB-231 cells and no *edg-1* mRNA was detected by RT-PCR or Northern analysis. SPP is rapidly taken up as the intact molecule and then degraded intracellularly to sphingosine. Thus, SPP may act intracellularly, and

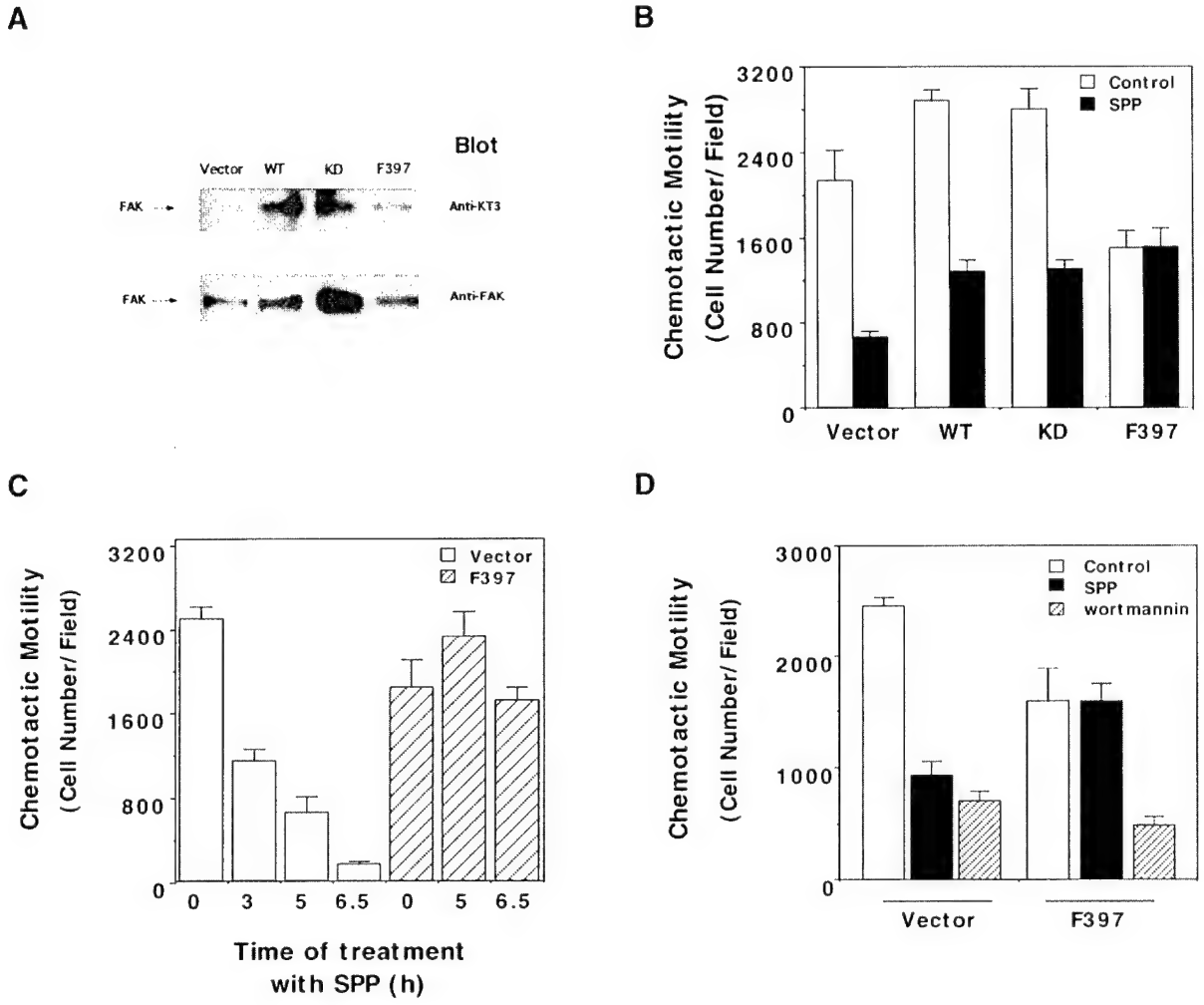

FIG. 7. SPP does not inhibit chemotaxis of MDA-MB-231 cells expressing autophosphorylation site-mutated FAK. (A) Cells overexpressing vector alone or wild type (WT), kinase-defective (KD), or autophosphorylation site-mutated (F397) FAK were lysed and analyzed by Western blotting with anti-KT3 (top) or anti-FAK mAb (bottom). (B) Chemotaxis of cells overexpressing vector alone or WT, KD, or mutated (F397) FAK was determined in the presence of SPP (15 μM) (filled bars) or vehicle (open bars). Cells were allowed to migrate for 4 h through polycarbonate filters coated with 5 μg of collagen IV and chemotaxis was measured as described under Materials and Methods. The effects of SPP were significantly different from control in all cells except in FAK-F397 cells as determined by Student's t test ($P < 0.05$). (C) Effect of duration of treatment with SPP on chemotaxis. Chemotaxis of control cells (open bars) or F397 FAK (hatched bars) was determined after 3 h migration. Cells were pretreated with SPP (15 μM) for 3.5 h (6.5 h) or 2 h (5 h) or with vehicle (0 h, 3 h). Vehicle (0 h) or SPP (15 μM) (3, 5, and 6.5 h) was present during the 3-h assay. (D) Control (vector) or FAK F397 cells were treated with vehicle (open bars), 15 μM SPP (filled bars), or 200 nM wortmannin (hatched bars) and chemotactic motility was measured.

not by binding to a putative cell surface receptor, to inhibit motility and invasion of MDA-MB-231 cells. This is consistent with the conclusion that SPP, formed in response to PDGF, plays an important role in PDGF-regulated motility of human arterial smooth muscle cells [16]. Moreover, we have recently demonstrated that several signaling pathways regulated by SPP, including calcium mobilization, activation of phosphlipase D, and tyrosine phosphorylation of FAK, were clearly independent of Edg-1 expression [23].

It was reported that an increased level of FAK expression is highly correlated with invasion potential of human tumor cells [44, 45], suggesting that FAK may be limiting for cell invasion. FAK has been indicated as playing a crucial role in cell migration events [36, 9, 46, 47]. Cultured fibroblasts isolated from FAK-deficient mice display reduced cell motility and enhanced focal adhesion contact formation [48]. In agreement, displacement of endogenous FAK from focal adhesions by microinjection of its carboxyl-terminal domain in endothelial cells resulted in decreased cell migration [46]. Overexpression of FAK, or a KD FAK mutant, promoted migration of CHO cells on fibronectin [9]. In agreement, we found that overexpression of wild-type or KD FAK slightly increased chemotactic motility of MDA-MB-231 cells.

Previously, in Swiss 3T3 fibroblasts, it was shown that SPP and sphingosine stimulated tyrosine phos-

phorylation of FAK, paxillin, and Crk [39], leading to stress fiber and focal contact formation [7, 15]. Yet the relevance of these observations to motility of Swiss 3T3 fibroblasts has not been determined. Extracellular matrix-dependent FAK activity was not altered in SPP-inhibited haptotactic motility of mouse B16 melanoma cells [43]. We found that SPP and sphingosine, but not ceramide, increased tyrosine phosphorylation of FAK in MDA-MB-231 cells with a concomitant increase in tyrosine phosphorylation of paxillin and Crk. This observation is intriguing since it has previously been shown that increased tyrosine phosphorylation of FAK correlates with increased cell motility induced by EGF [47] or PDGF [49]. However, NIH 3T3 cell lines overexpressing v-Src exhibit dramatically reduced migration rates and increased FAK phosphorylation [36]. Thus, it has been suggested that maximal migration requires optimal levels of FAK tyrosine phosphorylation [36].

Previously, Guan and co-workers demonstrated that overexpression of FAK, or a KD FAK mutant that was tyrosine phosphorylated and associated with Src, promoted migration of CHO cells on fibronectin [9]. This effect was dependent on FAK autophosphorylation at Y397 and subsequent binding of Src to this site. We found that overexpression of wild-type and KD FAK in MDA-MB-231 cells caused slightly increased chemotactic motility, without reducing the inhibitory effect of SPP. However, expression of autophosphorylation-site-mutated FAK abolished the effect of SPP on motility. In contrast, wortmannin, a fungal metabolite that binds directly to and inhibits the p110 subunit of PI3-kinase [40], markedly reduced chemotactic motility of these cell lines. Thus, the autophosphorylation site on FAK may play an important role in SPP-mediated signaling leading to decreased cell motility.

Autophosphorylation of FAK at Y397 leads to its association with Src, resulting in activation of both kinases (reviewed in [14]). The activated FAK/Src complex phosphorylates several substrates, including tensin, paxillin, and p130Cas. FAK phosphorylation and/or binding to paxillin and p130Cas may trigger downstream activation of MAP kinase by the adaptor protein Crk. Src association with FAK may also result in further FAK phosphorylation, forming a docking site for Grb2. However, recent results [11] demonstrate that p130Cas, but not Grb2, is a mediator of FAK-promoted CHO cell migration. FAK-induced p130Cas phosphorylation occurred when a kinase-defective FAK mutant but not F397 was expressed, indicating that Src may be the kinase that mediates phosphorylation of p130Cas [50]. This result also suggests that the inability of the Y397F FAK mutant to promote cell migration may be due to inefficient p130Cas phosphorylation and recruitment of signaling molecules. Moreover, it was demonstrated that the catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility [51]. Thus, it was proposed that the Src-FAK-linked activity induces focal adhesion turnover, facilitating cell movement. Further studies are needed to clarify the importance of Src in the motility-inhibiting effect of SPP. Since Y397 can also bind PI3-kinase, which might be important for motility [41, 42], the possibility exists that PI3-kinase is also important for SPP signaling. Moreover, our results indicate that the PI3-kinase pathway can also regulate the motility of MDA-MB-231 cells. However, overexpression of autophosphorylation site-mutated FAK did not abrogate the ability of the PI3-kinase inhibitor wortmannin to inhibit motility, suggesting that SPP signaling is clearly independent of the PI3-kinase pathway.

Inhibition of cell spreading by expression of the C-terminal domain of FAK is rescued by coexpression of Src or catalytically inactive FAK with concomitant enhancement of tyrosine phosphorylation of paxillin [52]. These results suggest that tyrosine phosphorylation of paxillin is a critical step in focal adhesion assembly. In this scenario, FAK may act as a "switchable adaptor" that recruits Src to phosphorylate paxillin, thus promoting focal adhesion assembly.

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SPHINGOSINE-1-PHOSPHATE INHIBITS MOTILITY OF HUMAN BREAST CANCER CELLS INDEPENDENTLY OF CELL SURFACE RECEPTORS

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[2] The abbreviations used are: BSA, bovine serum albumin; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate); C8-cer-1-P, N-octanoyl ceramide-1-phosphate; dihydro SPP, sphinganine-1-phosphate; DMS, N,N-dimethylsphingosine; EDG, endothelial differentiation gene; FBS, fetal bovine serum; FCM, fibroblast conditioned medium; GFP, green fluorescent protein; GPCR, G protein-coupled receptors; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; IMEM, Richter's Improved Minimal Essential Medium; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; SPC, sphingosylphosphorylcholine; RT-PCR, reverse transcriptase - polymerase chain reaction; SPP, sphingosine-1-phosphate.

ABSTRACT

Exogenous sphingosine 1-phosphate (SPP) inhibits chemotactic motility of several transformed cell lines. We have found that SPP at high micromolar concentrations decreased chemotaxis of estrogen-independent (MDA-MB-231 and BT 549) and of estrogen-dependent (MCF-7 and ZR-75-1) human breast cancer cells. Since SPP has been implicated as a lipid signaling molecule with novel dual intra and intercellular actions, it was of interest to determine whether the effect of SPP on chemotactic motility of human breast cancer cells is mediated intracellularly or through the recently identified EDG family of G protein-coupled SPP receptors. There was no detectable specific binding of [^{32}P]SPP to MDA-MB-231or MCF-7 cells; however, RT-PCR analysis revealed that both MDA-MB-231 and MCF-7 cells expressed moderate levels of EDG-3, neither expressed EDG-1, and EDG-5 mRNA was expressed in MCF-7, but not in MDA-MB-231 cells. In contrast to SPP, dihydro-SPP, which binds to and signals through SPP receptors, EDG-1, -3, and –5, had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. To further discriminate between intracellular and receptor-mediated actions of SPP, we used caged SPP, a photolyzable derivative of SPP which elevates intracellular levels of SPP after illumination. Caged SPP inhibited chemotactic motility of MDA-MB-231 cells only upon ultraviolet irradiation. In addition, in MCF-7 cells, overexpression of sphingosine kinase, the enzyme that produces SPP, inhibited chemotactic motility compared to vector-transfected cells, and markedly increased cellular SPP levels, in the absence of detectable secretion. Our results suggest that the inhibitory effect of SPP on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of SPP rather than through cell surface receptors.

INTRODUCTION

The sphingolipid metabolite, sphingosine 1-phosphate (SPP), has been shown to act on various signaling pathways, and to affect numerous biological functions (reviewed in (1)). SPP has been implicated as a lipid second messenger in mitogenesis and calcium mobilization and also antagonizes ceramide-mediated apoptosis (1). The resting level of SPP is very low in most cells. The enzyme which catalyzes its formation, sphingosine kinase, is increased by a wide variety of stimuli, including mitogens, such as PDGF and FBS (2-5), survival factors, such as NGF, bFGF (6), and TPA (7), as well as 1α,25-dihydroxyvitamin D3 (8), ligation of Fc receptors FcεRI (9) and FcγRI (10), muscarinic acetylcholine receptors (11), and the B subunit of cholera toxin (12). The elevation in SPP as a result of sphingosine kinase activation is relatively short-lasting, due to the action of two specific enzymes involved in its catabolism: SPP lyase and SPP phosphatase (13-17). Prevention of the increase in SPP by competitive inhibitors of sphingosine kinase selectively blocks cellular proliferation induced by PDGF and serum (2, 18), as well as FcεRI- and FcγRI-mediated calcium release from internal sources (9, 10), calcium influx induced by carbachol (11), and the cytoprotective effects of TPA, cAMP activators (19, 20), NGF (6), and vitamin D3 (8).

Although many studies indicate an intracellular site of action for SPP, pertussis toxin-sensitive G proteins have been shown to be involved in some of the signaling pathways regulated by SPP, suggesting that it might activate a receptor coupled to a G_i/G_o-protein (21). In agreement, low concentrations of SPP activated G_i protein-gated inwardly rectifying potassium channels only when SPP was applied to the extracellular face of guinea pig atrial myocytes (22). Also, nanomolar concentrations of SPP (EC_{50} = 2 nM) rapidly induced Rho-dependent neurite

retraction and cell rounding of mouse N1E-115 neurons (23) and platelet activation (24).

Recently, the G protein-coupled receptors EDG-1, EDG-3 and EDG-5 were identified as high affinity receptors for SPP (25-29). EDG-1 causes morphogenetic differentiation in response to SPP when expressed in HEK293 cells (25), while EDG-5, and to a lesser extent EDG-3, cause cell rounding in HEK293 cells and neurite retraction in PC12 cells (28). Binding of SPP to Edg-1, -3, and -5, stimulates different Gαs and βγ dimers to signal through cAMP, phospholipase C, Ras, mitogen-activated protein kinase, Rho, and several protein tyrosine kinases (25, 26, 29-32). Collectively these studies suggest that SPP is capable of acting as a second messenger to regulate cell proliferation and survival and as a first messenger through the EDG family of GPCR to regulate diverse biological responses.

Migration of cells is important in a variety of normal physiological processes, including embryogenesis, reproduction, inflammation, and wound healing. Moreover, cell motility plays a notable role in pathophysiological processes important for malignant progression such as metastasis. Cell migration is regulated by both expression of adhesion molecules and deposition of basement membrane or matrix proteins and soluble extracellular molecules interacting with specific cell surface receptors (33).

Previously, many studies have shown that exogenous SPP inhibits the chemotactic motility of various cancer cells at very low nanomolar concentrations (10-100 nM) (34-36). SPP also inhibits integrin-dependent motility (haptotactic motility) of mouse melanoma B16 cells by inhibiting actin nucleation and pseudopodium formation, without reducing integrin-dependent adhesion to the extracellular matrix (35). Furthermore, SPP immobilized on controlled pore glass beads inhibits motility of mouse melanoma cells (37), indicating that this

effect may be mediated through cell surface receptors. In contrast, we previously found that inhibition of chemotactic motility of human breast cancer MCF-7 and MDA-MB-231 cells requires micromolar concentrations of SPP (38). Thus, it is of interest to determine whether SPP acts extracellularly as a ligand for cell surface receptors or intracellularly as a second messenger to inhibit chemotactic motility of human breast cancer cells.

The use of exogneously administered SPP is a major limitation of most previous studies since this approach cannot distinguish between receptor-mediated and intracellular effects as SPP is readily taken up by cells in culture (2, 26, 39). In this study, we demonstrated that motility of human breast cancer cells was inhibited when intracellular levels of SPP were increased either after photolysis of caged SPP or by overexpression of sphingosine kinase, both approaches bypass cell surface receptors. Our results suggest that SPP can inhibit motility through intracellular actions.

MATERIALS AND METHODS

Materials. SPP, dihydrosphingosine-1-phosphate (dihydro-SPP), sphingosine, and N,N-dimethylsphingosine (DMS) were purchased from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). SPP was greater than 99% pure by thin layer chromatography analysis. C8-SPP, C8-ceramide-1-phosphate, octyl β-D-glucopyranoside and bovine brain ceramides (type IV) were from Calbiochem (La Jolla, CA). CyclicSPP was purchased from Alexis Biochemicals (San Diego, CA). Other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). Media, serum and supplements were from Biofluids Inc. (Rockville, MD); insulin and transferrin from Collaborative Research (Lexington, MA); bovine serum albumin, alkaline phosphatase

(type VII-T) and Quik-fix kit from Sigma Chemical Co. (St. Louis, MO); and G418 from Mediatech (Herndon, VA). Collagen type IV was purchased from Collagen Corp. (Palo Alto, CA). Polycarbonate filters were from Poretics (Livermore, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL).

Cell culture. The human breast cancer cell lines MDA-MB-231, MCF-7, BT 549, and ZR-75-1 were obtained from the Cell Culture Core Resource, Lombardi Cancer Center (Washington, D.C.). Cells were maintained in Richter's Improved Minimal Essential Medium (IMEM) supplemented with 10% fetal bovine serum (FBS). Unless indicated, cells were seeded at 1.8 x 10^4 cells/cm^2. Twenty four hours prior to experiments, the media was changed to serum-free IMEM without phenol red supplemented with 2 mM HEPES, 1% (v/v) non-essential amino acids, 1% (v/v) trace elements, 0.4% (v/v) insulin, transferrin, and selenium, 0.2% (w/v) fibronectin, and 1% (v/v) vitamins (38).

Chemotaxis Assay. Boyden chamber chemotactic motility assays were carried out essentially as previously described (38). Polycarbonate filters (13 mm diameter, 12 μm pore size) were coated with collagen IV (5 μg/filter) and then placed into the lower chamber. Collagen IV coating promotes uniform attachment to and migration across the filter, without formation of a barrier. Fibroblast conditioned medium (FCM), obtained by incubating confluent NIH 3T3 cells for 48 h with IMEM supplemented with L-ascorbic acid (50 μg/ml), was placed in the lower chamber as chemoattractant. Cells were harvested by trypsinization, washed twice with IMEM containing 0.1% BSA, and 0.75 x 10^5 cells were added to the upper chamber and treated as indicated. The chambers were incubated in a humidified incubator at 37°C in 5% CO_2/95% air for 4 h or 24 h as indicated. The cells which traversed the filter and spread on the lower surface of the filter were

fixed and stained with Quik fix kit. Nonmigratory cells on the upper membrane surface were removed with a cotton swab. The number of migratory cells per membrane was determined using light microscopy with a 10X objective. Each data point is the average number of cells in four random fields counted twice. Data are expressed as the means of counts from three individual wells ± SD.

SPP Binding Assay. [^{32}P]SPP was synthesized enzymatically using recombinant sphingosine kinase as previously described (26). The specific activity of [^{32}P]SPP was 6 x 10^6 cpm/pmol. Cells (10^5) were incubated with 1 nM [^{32}P]SPP in 200 μl binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 15 mM NaF, 2 mM deoxypyridoxine, 0.2 mM PMSF, 1 μg/ml aprotinin and leupeptin) for 30 minutes at 4°C. Cells were washed twice with 200 μl, ice cold binding buffer containing 0.4 mg/ml fatty acid-free BSA, resuspended in PBS and specific binding of [^{32}P]SPP was determined in the presence of 1 μM unlabeled SPP (26).

Reverse Transcriptase (RT)-PCR. Total RNA was isolated from cells using TRIZOL reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions and treated with RNase-free DNase I (RQ-1, Promega, Madison, WI) to eliminate contaminating DNA. Reverse transcription was performed using MULV-RT (Perkin Elmer, Branchburg, NJ) for 15 min at 42°C. The primers (Life Technologies) used for PCR amplification were 5'-GATATCATCGTCCGGCATTAC and 5'-ACCCTTCCCAGTGCATTGTTC for *EDG-1* (40); 5'-CACTCAGCAATGTACCTGTTCC and 5'-AACACCCAGTACGATGGTGAC for *EDG-5* (41, 42); and 5'-GACTGCTCTACCATCTTGCCC and 5'-GTAGATGACAGGGTTCATGGC for *EDG-3* (43). PCR reactions were performed for 30 cycles with denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, and elongation at 72°C for 50 sec. The PCR products were

analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Expression of Sphingosine Kinase. SPHK1a was subcloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) to express it with an N terminal c-myc epitope tag by PCR using a 5' primer with a BamH1 restriction site (5'-GAGGGATCCGAACCAGAATGCCCTCGAGGA-3'), and as the 3' primer, the last 21 nucleotides of the SPHK1a sequence with an EcoRI overhang (5'-GAGGAATTCTTATGGTTCTTCTGGAGGTGG-3'). MCF-7 cells were seeded at 4 x 10^6 in 150 mm dishes and after 18 h, transfected with vector (c-myc-pcDNA3) or with the vector construct containing a sphingosine kinase insert (c-myc-pcDNA3-SPHK1a) (44) using Lipofectamine-Plus (Gibco) according to manufacturer's instructions. In some cases, cells were co-transfected with pCEFLGFP, which encodes the green fluorescent protein, and visualized with a fluorescence microscope as a measure of transfection efficiency. Stable transfectants were selected in the presence of 0.5 g/L G418 (44).

Western Blotting. Transfected cells were lysed in a buffer containing 0.1% CHAPS, 10 mM HEPES, 2 mM EDTA, 5 mM DTT, 1 mM PMSF, and 10 μg/ml of pepstatin A, leupeptin and aprotinin. Lysates were centrifuged at 14,000 x g for 15 min and 20 μg protein from the supernatants were separated by SDS-PAGE prior to immunoblotting with a monoclonal anti-c-myc (9E10) antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized with Super Signal chemiluminescent reagent (Pierce, Rockford, IL) using horseradish peroxidase conjugated anti-mouse IgG (1:5000).

Sphingosine Kinase Assay. Transfected cells were harvested and lysed by repeated freeze-thawing in buffer A (20 mM Tris (pH 7.4), 20% glycerol, 1 mM ß-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM ß-glycero-phosphate, 15 mM NaF, 10 μg/ml

leupeptin, aprotinin and soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine). Sphingosine kinase activity was determined in the presence of 50 μM sphingosine, 0.25% Triton X-100, and [^{32}P]ATP (10 μCi, 20 mM) containing $MgCl_2$ (200 mM) in buffer A as previously described (45). The labeled SPP was separated by TLC on silica gel G60 with chloroform/methanol/acetone/acetic acid/water (10:4:3:2:1, v/v) and visualized by autoradiography. Radioactive spots corresponding to authentic SPP were identified as described (39) and quantified with a Molecular Dynamics Storm Phosphoimager (Sunnyvale, CA). Sphingosine kinase specific activity was expressed as pmol of SPP formed per min per mg of protein.

Extraction of Lipids. Transfected cells were washed with PBS and scraped in 1 ml 25 mM HCl/methanol. Lipids were extracted with 2 ml of chloroform/1M NaCl (1:1, v/v) plus 100 μl 3N NaOH and phases separated (46). Phospholipid, ceramide and sphingosine levels were determined in aliquots of the organic layer, while SPP levels were determined from aqueous phase extracts. Extraction of SPP from the medium of cells labeled with 40 μCi [$^{32}P_i$] for 48 hours was performed similarly. To 1 ml of medium, 2 ml of chlorform/methanol (1:1 v/v) plus 100 μl 3N NaOH was added and phases were separated. The aqueous phase containing SPP was then acidified with 50 μl conc. HCl and re-extracted twice with 1 ml of chlorform, and organic fractions were pooled (46).

Measurement of SPP Levels. SPP levels were measured essentially as described (46). Briefly, buffer B (200 mM Tris-HCl, pH 7.4, 75 mM $MgCl_2$ in 2 M glycine, pH 9.0) and 50 U of alkaline phosphatase were added to the aqueous phase containing extracted SPP. After incubating 1 h at 37°C, 50 μl conc. HCl were added and sphingosine was extracted and quantitated with

sphingosine kinase as described (46). For each experiment, known amounts of SPP were used to generate a standard curve.

Measurement of Sphingosine Levels. Sphingosine was measured by minor modifications of a previously described method (47). Briefly, aliquots of lipid extracts containing ≥ 50 nmol of total phospholipid were dried under nitrogen, resuspended in buffer A containing 0.25% Triton X-100, and sphingosine converted to SPP with sphingosine kinase was measured as described (47). For each experiment, known amounts of sphingosine were used to generate a standard curve.

Measurements of Ceramide Levels. Mass amounts of ceramide in cellular extracts were measured by the diacylglycerol kinase enzymatic method exactly as described (6).

Measurement of Total Cellular Phospholipids. Total phospholipids in cellular lipid extracts were quantified as previously described (48).

RESULTS

SPP Inhibits Chemotactic Motility of Human Breast Cancer Cells

In agreement with our previous report (38), we found that SPP inhibits chemotaxis of human breast cancer cells (Fig 1A). SPP (10 μM) decreased chemotaxis of estrogen-independent MDA-MB-231 and BT 549 human breast cancer cells by 45% and 28%, respectively. Treatment of estrogen-dependent human breast cancer cells with SPP decreased chemotaxis by 87% (MCF-7) and 45% (ZR-75-1). In addition, treatment with high μM concentrations of SPP and sphingosine also slightly, but significantly, inhibited random motility of MDA-MB-231 cells (Fig 1B).

Expression of SPP Receptors in MDA-MB-231 and MCF-7 Cells

It has recently been reported that SPP binds specifically and with high affinity to the GPCRs, EDG-1 (25), EDG-3 and EDG-5 (H218/AGR16) (28). We therefore examined expression of SPP receptor mRNAs in MCF-7 and MDA-MB-231 cells using RT-PCR analysis. The entire open reading frame of each of the three SPP receptors is encoded within a single exon (49, 50), and it is not possible to design PCR primers which span an intron junction. Therefore, controls without reverse transcriptase were performed in all cases to ensure that the product observed was not derived from genomic DNA (Fig. 2). Expression of *EDG*-3 mRNA was detected in MDA-MB-231 cells; however, neither *EDG*-1 nor *EDG*-5 mRNA could be detected. MCF-7 cells expressed *EDG*-3 as well as *EDG*-5, but similarly to MDA-MB-231 cells, did not express *EDG*-1. MCF-7 cells stably overexpressing *EDG*-1 were included as a positive control. Digestion of the human RT-PCR products with restriction enzymes gave fragments of expected sizes confirming their identity (data not shown).

To confirm that SPP receptors were constitutively expressed in MCF-7 and MDA-MB-231 cells, we attempted to measure specific binding of [^{32}P]SPP. In both cell lines, total binding of 1 nM [^{32}P]SPP was low and was not significantly reduced by addition of 1000-fold excess of unlabeled SPP, indicating very low expression of specific SPP receptors by these cells. The failure to detect specific SPP binding is not a result of improper binding conditions, since in MCF-7 cells overexpressing EDG-1, significant specific SPP binding could be detected (10.3 ± 0.6 fmol/10^5 cells).

Effects of SPP Analogs on Chemotaxis

Low nanomolar concentrations of SPP have been shown to inhibit chemotaxis of human

melanoma cells (34, 37). In contrast, although high concentrations of SPP markedly inhibit chemotaxis of MDA-MB-231 cells, low concentrations of SPP (from 10 nM to 1 μM) had no significant effects (Fig. 3A). Previously, we found that similar to SPP, high concentrations of sphingosine also inhibit chemotaxis of these cells (38). Thus, it was of interest to compare the effects of several other structurally-related SPP analogs (Fig. 3A). None of the other SPP analogs tested, including the short-chain SPP analog C8-SPP, N-octanoyl ceramide-1-phosphate (C8-Cer-P), cyclic-SPP, or caged-SPP (see below), significantly inhibited motility of MDA-MB-231 cells. Because of the structural similarities between LPA and SPP, and because they have been reported to bind to the homologous receptors, EDG-2 and EDG-4 (29), we also examined the effect of LPA. Recently it has been suggested that LPA is a low affinity ligand for EDG1 (51). However, LPA, even at concentrations as high as 10 μM, had no significant effect on chemotaxis of MDA-MB-231 cells (Fig. 3A).

EDG-1, -3, and -5, bind SPP with high specificity. Dihydro-SPP, which is similar to SPP but lacks the trans double bond, binds to and signals through all three SPP receptors with similar potency to SPP (25, 26, 28). Thus, if inhibition of chemotactic motility is mediated through one or more of these SPP receptors, dihyro-SPP should mimic the effect of SPP. However, 10 μM dihydro-SPP had no significant effect on chemotactic motility of MDA-MB-231 or MCF-7 cells (Fig. 3B). Collectively, these data suggest that the ability of SPP to inhibit chemotaxis may not be related to binding to EDG cell surface receptors.

Inhibition of Chemotaxis by Caged SPP

To determine whether SPP formed intracellularly regulates cell motility, it is useful to be able to experimentally increase intracellular SPP bypassing cell surface receptors. Recently, we

synthesized a photolyzable derivative of SPP, termed caged SPP, which is taken up by cells and upon ultraviolet irradiation, is photolysed to form SPP and *o*-nitrosoacetophenone (52). The introduction of caged SPP into cultured cells permit rapid and controlled elevation of intracellular SPP levels upon illumination (data not shown). The effect of caged SPP photolysis on chemotactic motility of MDA-MB-231 cells was compared to that of exogenously added SPP (Fig. 4). Exogenous SPP (10 μM) inhibited chemotactic motility of MDA-MB-231 cells by 50% while caged SPP (5 or 10 μM) had no significant effect on chemotactic motility in non-illuminated cells or in cells illuminated prior to its addition. However, ultraviolet irradiation of caged SPP-loaded cells caused strong inhibition of chemotaxis, whereas ultraviolet irradiation alone had no effect (Fig. 4), nor did it alter the inhibitory effect of exogenous SPP. To exclude the possibility that the by-product of caged SPP photolysis, *o*-nitrosoacetophenone, may affect chemotaxis, motility of cells treated with caged cyclic-SPP was measured. Unlike caged SPP, caged cyclic-SPP had no effect on chemotaxis of MDA-MB-231 cells with or without ultraviolet irradiation (Fig. 4). Moreover, incubation of cells with *o*-nitrosoacetophenone had no effect on chemotaxis, even when cells were incubated for several hours at very high concentrations (100 μM, data not shown).

Overexpression of Sphingosine Kinase Decreases Chemotactic Motility of MCF-7 Cells

As an alternative method to increase the level of intracellular SPP, MCF-7 cells were transfected with a murine sphingosine kinase expression vector (44). Transient expression of sphingosine kinase in MCF-7 cells decreased chemotaxis towards FCM (Fig. 5A). Moreover, although the number of migrating cells was much lower in the absence of chemoattractant, transient expression of sphingosine kinase also significantly reduced this random motility (Fig.

5A).

To further examine the effect of overexpression of sphingosine kinase on chemotaxis and to correlate this with protein expression, MCF-7 cells were transfected with c-myc-tagged sphingosine kinase and selected on the basis of G418 resistance. Pools of stably transfected cells were used in order to avoid potential phenotypic changes due to selection and propagation of clones derived from single individual cells. Stable expression of sphingosine kinase decreased chemotactic motility of MCF-7 cells by 30% and 46% after 4 h and 24 h, respectively (Fig. 5B). Sphingosine kinase activity in the cytosolic fraction of these transfected cells was elevated by 36 fold over control vector-transfected cells (Table I), whereas SPP levels were elevated only by 3.3 fold (Table I). This is in agreement with previous results (44), where the large fold increase in transfected sphingosine kinase activity measured *in vitro* did not correspond with the fold increase in SPP levels. To further examine the correlation between expression of sphingosine kinase and increased SPP levels with inhibition of chemotaxis, we generated a separate independent pool of transfected cells which express even higher levels of sphingosine kinase (Table I). Western blot analysis of the cytosolic fractions of these cells using anti-c-myc antibody revealed a band migrating at the expected molecular weight of c-myc-tagged sphingosine kinase which was absent in vector transfected cells (Fig. 6A, Inset). Interestingly, although expression of sphingosine kinase in this second pool of transfected MCF-7 cells resulted in even more marked increase in mass levels of SPP (Fig. 6B), there were no concomitant changes in the other sphingolipid metabolites, sphingosine (Fig. 6C) or ceramide (Fig. 6D). Nevertheless, chemotaxis of the MCF-7 cells with higher sphingosine kinase expression and cellular SPP levels was more strongly inhibited (Fig. 6E).

Because it has been suggested that SPP inhibits melanoma cell motility through an extracellular action by specific binding to cell surface receptors (37), it was of interest to determine whether MCF-7 cells overexpressing sphingosine kinase, which have increased cellular levels of SPP, can secrete SPP. We have recently developed a sensitive assay for the measurement of SPP (46) that is able to detect as little as 1 pmol SPP. Using this assay, we were unable to detect secretion of SPP into the media by either of the stable pools of sphingosine kinase transfected MCF-7 cells (Table I), suggesting that SPP is not released by these cells in appreciable amounts. To increase the sensitivity of detection of secreted SPP, we also labeled cells to isotopic equilibrium with [^{32}P]P_i and analyzed the labeled SPP in cells as well as in the medium. Despite the large increases in [^{32}P]SPP detected in MCF-7 cells overexpressing sphingosine kinase, there was no detectable labeled SPP released into the medium. Based on the sensitivity of these methods, we estimate that the concentration of SPP in the extracellular media is ≤0.4 nM, a concentration well below the K_d for binding of SPP to its EDG receptors (26, 28).

DISCUSSION

SPP has been shown to function both intracellularly as a second messenger and at the cell surface through specific SPP receptors to regulate numerous biological processes (29, 53). Previously, based on addition of SPP to cells, many studies have suggested that SPP inhibits cell motility and chemotaxis by binding to a putative cell surface receptor (reviewed in (37)). Recently, EDG-1 was identified as a high affinity (K_d = 8 nM), specific receptor for SPP (25). Two related receptors, EDG-3 and EDG-5 (also known as AGR16/H218), also bind SPP with low nanomolar affinities (28). However, it has not yet been determined whether EDG-1, -3, or

–5 play a role in mediating the effects of exogenous SPP on inhibition of motility.

SPP has been shown to inhibit cell motility, chemoinvasion, and haptotactic motility (35) of human B16 melanoma cells in a low concentration range (10 to 100 nM) (34). These effects appear to be mediated through a cell surface receptor, since SPP immobilized on glass beads, which cannot traverse the cell membrane, mimicked the effects of SPP (37). SPP also inhibited the chemotactic motility and trans-endothelial migration of human neutrophils (36) and PDGF-induced chemotaxis of aortic smooth muscle cells (3) at nanomolar concentrations. In contrast, micromolar concentrations of SPP were necessary to inhibit chemotactic motility of human breast cancer cell lines (MCF-7 and MDA-MB-231) (38) and human HT1080 fibrosarcoma cells (34). It was thus important to determine whether inhibition of breast cancer cell motility by SPP is mediated intracellularly or through a cell surface receptor.

Surprisingly, in this study, several lines of evidence indicated that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells through an intracellular action rather than by signaling through cell surface receptors. First, SPP had no effect on motility of MDA-MB-231 cells at concentrations below 1 μM, approximately two orders of magnitude higher than the K_d for binding of SPP to EDG-1, -3, or –5 (26, 28). Second, although MDA-MB-231 cells express SPP receptor EDG-3 mRNA, and MCF-7 cells express EDG-3 and EDG-5, no specific SPP binding could be detected to either MCF-7 or MDA-MB-231 cells, indicating that either the receptor proteins are not present on the cell surface or that they are expressed at very low levels. Thirdly, dihydro-SPP, which binds to and signals through all three SPP receptors (26, 28 and Van Brocklyn and Spiegel, unpublished observations), had no effect on chemotactic motility of MDA-MB-231 or MCF-7 cells. In agreement, it was previously reported that dihydro-SPP did

not affect chemotactic motility of human neutrophils (36). Moreover, sphingosine, which is rapidly taken up by cells and converted intracellularly to SPP by sphingosine kinase, also inhibits chemotaxis of MCF-7 and MDA-MB-231 cells (38).

Additionally, two independent approaches were used to elevate intracellular SPP bypassing cell surface SPP receptors: treatment of cells with caged SPP, which is taken up by cells and forms SPP intracellularly upon UV irradiation (52); and overexpression of sphingosine kinase, the enzyme which forms SPP within cells. After UV photolysis of intracellular caged SPP, chemotactic motility was inhibited to the same extent as after treatment with exogenous SPP. Overexpression of sphingosine kinase by transfection in MCF-7 cells led to increased intracellular SPP and drastically inhibited chemotactic motility as well as random motility. Although the intracellular levels of SPP were elevated 3 to 4.6 fold in stably transfected cells, no detectable amounts of SPP were released into the medium. Thus, it seems highly unlikely that SPP inhibits motility of breast cancer cells by binding to cell surface receptors.

Although we have now shown that elevation of the intracellular levels of SPP inhibits motility of human breast cancer cells, our studies cannot exclude the possibility that in other cell types, the mode of action of SPP may vary depending on the expression of different SPP receptors and the signaling pathways that the receptors couple to in different cell types. It is possible that SPP could effect the same biological response through separate mechanisms even in the same cells. For example, microinjected SPP is mitogenic for Swiss 3T3 cells, however, to a lesser degree than exogenously added SPP (26). Although the mitogenic effect of microinjected SPP is insensitive to pertussis toxin treatment, the effect of exogenous SPP is partially inhibited by pertussis toxin, decreasing the response to approximately the same level as that induced by

microinjection (26). These results suggested that in Swiss 3T3 cells, which express all three known SPP receptors, the mitogenic effect of SPP is due to the additive contributions of receptor-mediated responses and intracellular actions. Thus, it is possible that cell motility could also be regulated by both intracellular actions of SPP, as in breast cancer cells, and possibly by receptor-mediated actions of SPP, as in melanoma cells.

The mechanism of the inhibitory effect of SPP on motility has not yet been elucidated. It is possible that the inhibitory effect of SPP on chemotactic motility may be mediated by focal adhesion kinase (FAK) and Rho. Activation of Rho results in the formation of actin stress fibers and focal adhesions (54-56) and it is well established that cytoskeletal changes regulated by Rho GTPases form the basis for the coordination of cell motility (57, 58). SPP induces tyrosine phosphorylation of FAK and actin stress fiber formation, in a Rho-dependent manner (59). Although the exact connection between the expression and tyrosine phosphorylation of FAK and cell motility is not completely understood, it has been suggested that maximal migration requires optimal levels of FAK tyrosine phosphorylation (60) and inhibition of FAK signaling in focal adhesions decreases cell motility (61). Intriguingly, it was found recently that GRAF, the GTPase regulator associated with FAK, that preferentially stimulates the GTPase activity of RhoA and Cdc42, can regulate cytoskeletal changes induced by SPP (62). In Swiss 3T3 cells, where GRAF is not expressed at detectable levels, GRAF overexpression inhibited SPP-induced, Rho-mediated stress fiber formation. Conversely, in PC12 cells which express high levels of GRAF, overexpression of GRAF stimulated Rho-mediated neurite retraction induced by SPP (62). These results may suggest that an optimal level of Rho activity is required for maximal cell motility. Another possibility is that the effect of SPP on motility is related to regulation of

cytosolic free calcium. It has been shown that large (micromolar) global increases in the cytosolic free calcium concentration in neutrophils are usually associated with termination of chemotaxis (63). Recently it was found that cells move forward during the phase of transient calcium elevation and remain stationary during the troughs (64). Consequently, changes in the frequency of Ca^{2+} fluctuations directly affect cerebellar granule cell movement, i.e. reducing the frequency of calcium fluctuations slows down the speed of cell movement (64). Interestingly, SPP has been found to elicit a non-oscillatory increase of intracellular free calcium in CG4, NIH 3T3 and PDGF receptor-transfected PC-12 cells (65). Moreover, inhibition of sphingosine kinase with DL-threo-dihydrosphingosine, significantly reduced the percentage of cells responding to PDGF exposure with calcium oscillations in transformed oligodendrocytes (66). Thus, it is possible that SPP inhibits chemotactic motility of MDA-MB-231 and MCF-7 cells by inducing a non-oscillatory increase in intracellular free calcium. Further studies are needed to clarify the importance of different signaling pathways for controlling motility that are regulated by SPP. Because cell movement is considered as an important step in invasion and metastasis of cancer cells, the finding that endogenous SPP regulates cell migration and chemotactic signaling may have substantial biological ramifications.

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TABLE I. SPHINGOSINE KINASE ACTIVITY AND SPP LEVELS IN POOLED CLONES OF MCF-7 CELLS STABLY EXPRESSING SPHINGOSINE KINASE

	Pool 1	Pool 2
Sphingosine Kinase Activity (fold increase)	36 ± 3	115 ± 7
Cellular SPP Levels (fold increase)	3.3 ± 0.4	4.6 ± 0.2
Secreted SPP (pmol/ml)	ND	ND

Pooled clones of MCF-7 cells stably transfected with c-myc-pCDNA3-SPHK1a or vector alone (c-myc-pCDNA3) were washed and incubated in serum-free medium overnight. Cytosolic fractions were prepared and sphingosine kinase activity measured as described in Materials and Methods. Data are expressed as fold increases compared to respective controls and are means ± SD of triplicate determinations from a representative experiment. Cellular and secreted SPP levels were measured after 24 h in serum-free medium. ND, not detected (≤ 0.4 pmol/ml).

FIGURE LEGENDS

Figure 1. SPP decreases motility of human breast cancer cells. (A) Effect of SPP on chemotaxis. MDA-MB-231, MCF-7, ZR-75-1 and BT-549 cells were allowed to migrate through polycarbonate filters coated with collagen IV for 4 h in the absence (open bars) or presence of SPP (10 μM) (filled bars). FCM was present in the lower chamber as a chemoattractant and chemotaxis was measured as described in Materials and Methods. **(B) Effect of SPP on random motility.** MDA-MB-231 cells were incubated in the absence or presence of SPP (10 μM) or sphingosine (Sph, 15 μM) and random motility was measured in the absence of FCM. The results are from a representative experiment repeated three times. All treatments were significantly different from controls as determined by Student's t-test ($p \leq 0.05$).

Figure 2. Detection of EDG receptor mRNA. RT-PCR analysis of GAPDH (G), *EDG-1* (1), *EDG-3* (3) and *EDG-5* (5) mRNA expression was performed on RNA isolated from MCF-7 MDA-MB-231 and MCF-7 cells stably expressing myc epitope-tagged *EDG-1* (MCF-7*EDG-1*), without (-) or with (+) MULV-RT (RT) as described in Materials and Methods. Similar results were obtained in two independent experiments.

Figure 3. Effect of structurally related SPP analogs on chemotaxis. (A) MDA-MB-231 cells were treated without or with the indicated concentrations of SPP, 10 μM C8-SPP, C8-ceramide-1-phosphate (C8-Cer-P), cyclic-SPP, or LPA and chemotaxis was determined. **(B) Differential effects of dihydro-SPP and SPP on motility.** MDA-MB-231 or MCF-7 cells were treated without or with 10 μM SPP or dihydroSPP (DH-SPP) and chemotactic motility was determined. The results are from a representative experiment repeated at least two times. The asterisks indicate significant differences from untreated controls as determined by Student's t-test ($p \leq 0.05$).

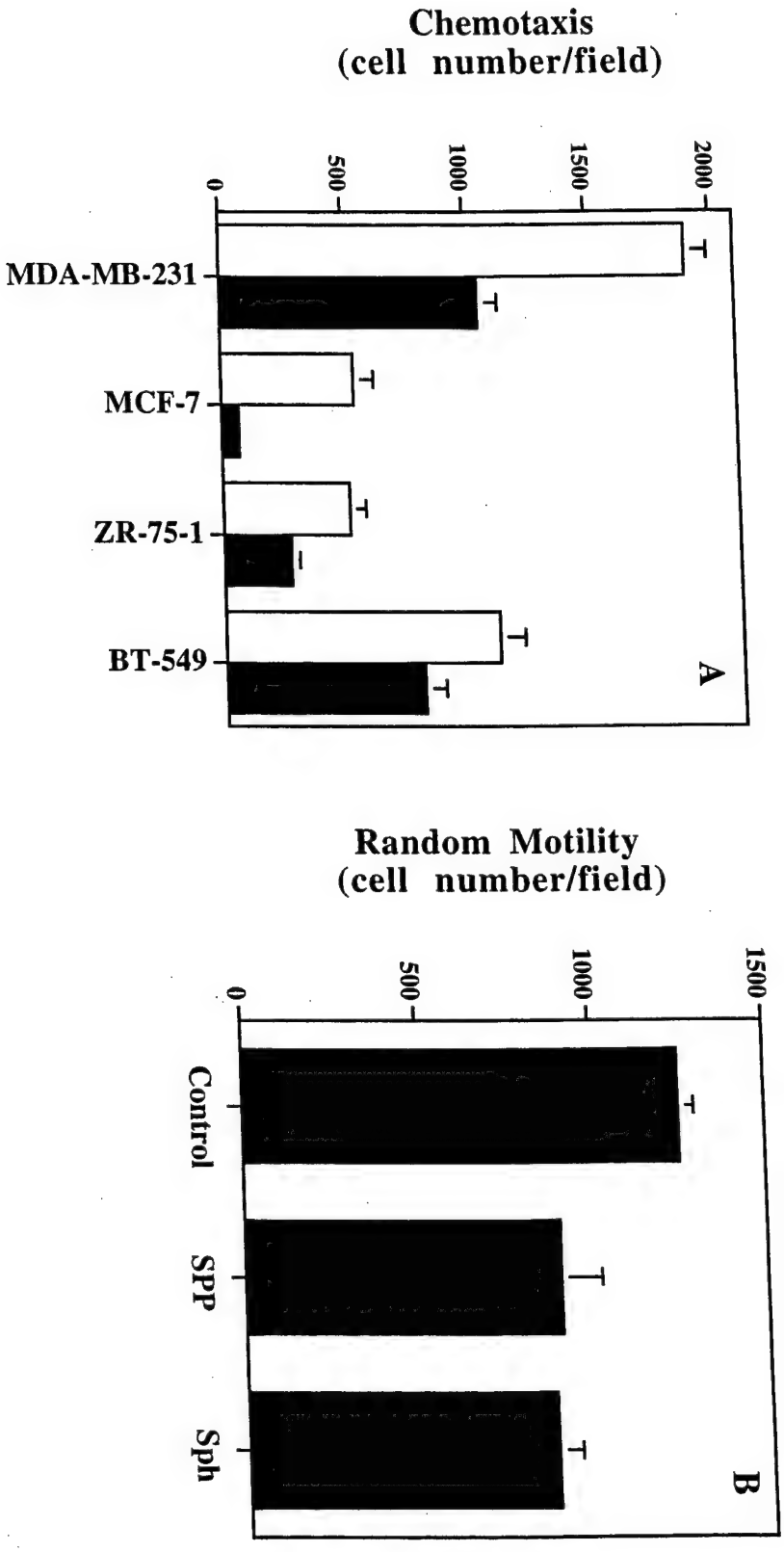
Figure 4. Effect of caged SPP on chemotactic motility. MDA-MB-231 cells were incubated without (control) or with the indicated concentrations of caged SPP, SPP, cyclic SPP, or caged cyclic SPP for 2 h. Cells were then collected, washed twice with IMEM containing 4 mg/ml fatty acid-free BSA and chemotaxis was measured without (filled bars) or with UV irradiation for 30 sec (open bars). Data are means ± SD of triplicate determinations. The results are from a representative experiment repeated three times. The asterisks indicate significant differences from controls as determined by Student's t-test ($p \leq 0.05$).

Figure 5. Overexpression of sphingosine kinase decreases chemotactic motility of MCF-7 cells. (A) MCF-7 cells transiently co-transfected with pCEFLGFP and either vector c-myc-pcDNA3 (open bars) or c-myc-pcDNA3-SPHK1a (filled bars). 48 h later cells were trypsinized, washed with serum free media and allowed to migrate in a Boyden chamber for 4 h toward either media alone (IMEM) as a measure of random motility or toward FCM as chemoattractant to measure chemotaxis. **(B)** Pooled clones of MCF-7 cells stably expressing c-myc-pcDNA3 (open bars), or c-myc-pcDNA3-SPHK1a (filled bars) were allowed to migrate toward FCM for 4 h or 24 h and chemotaxis was measured. The asterisks indicate significant differences from untreated controls as determined by Student's t-test ($p \leq 0.05$).

Figure 6. Changes in mass levels of sphingolipid metabolites in pooled clones of MCF-7 cells overexpressing sphingosine kinase and corresponding inhibition of motility. Pooled clones of MCF-7 cells expressing c-myc-pCDNA3-SPHK1a (filled bars) or vector alone (c-myc-pCDNA3, open bars) were washed and incubated in serum-free medium overnight. Sphingosine kinase activity **(A)**, mass levels of SPP **(B)**, sphingosine **(C)**, and ceramide **(D)** were measured as described in Materials and Methods. Data are means ± SD of triplicate

determinations from a representative experiment. (A, Insert) Western blot of MCF-7 cell lines expressing c-myc-pcDNA3 (lane 1) or c-myc-SPHK1a (lane 2), respectively. Equal amounts of protein (20 μg) were analyzed by immunoblotting following SDS-PAGE. The arrowhead indicates the migration of myc-tagged SPHK1a. **(E)** These transfected cells were allowed to migrate toward either media alone (IMEM) or FCM for 4 h and chemotaxis was measured. Data are means ± SD of triplicate determinations.

Fig 1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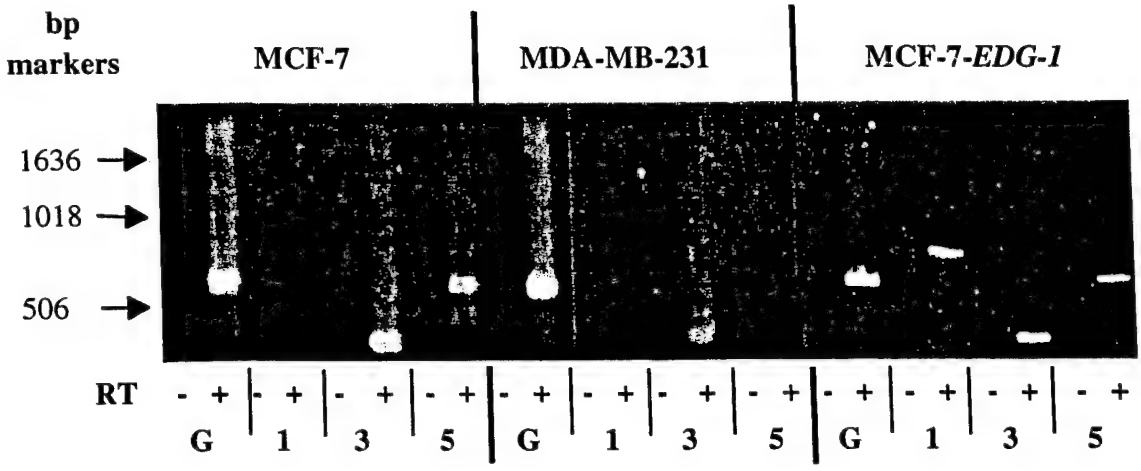
500
1000
1500
2000
MDA-MB-231
MCF-7
ZR-75-1
BT-549
A
Random Motility
(cell number/field)
0
500
1000
1500
Control
SPP
Sph
B

Fig 2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MDA-MB-231
MCF-7-EDG-1
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1018
506
RT
- + - + - + - + - + - + - + - + - + - + - + - +
G 1 3 5 G 1 3 5 G 1 3 5

Fig 3

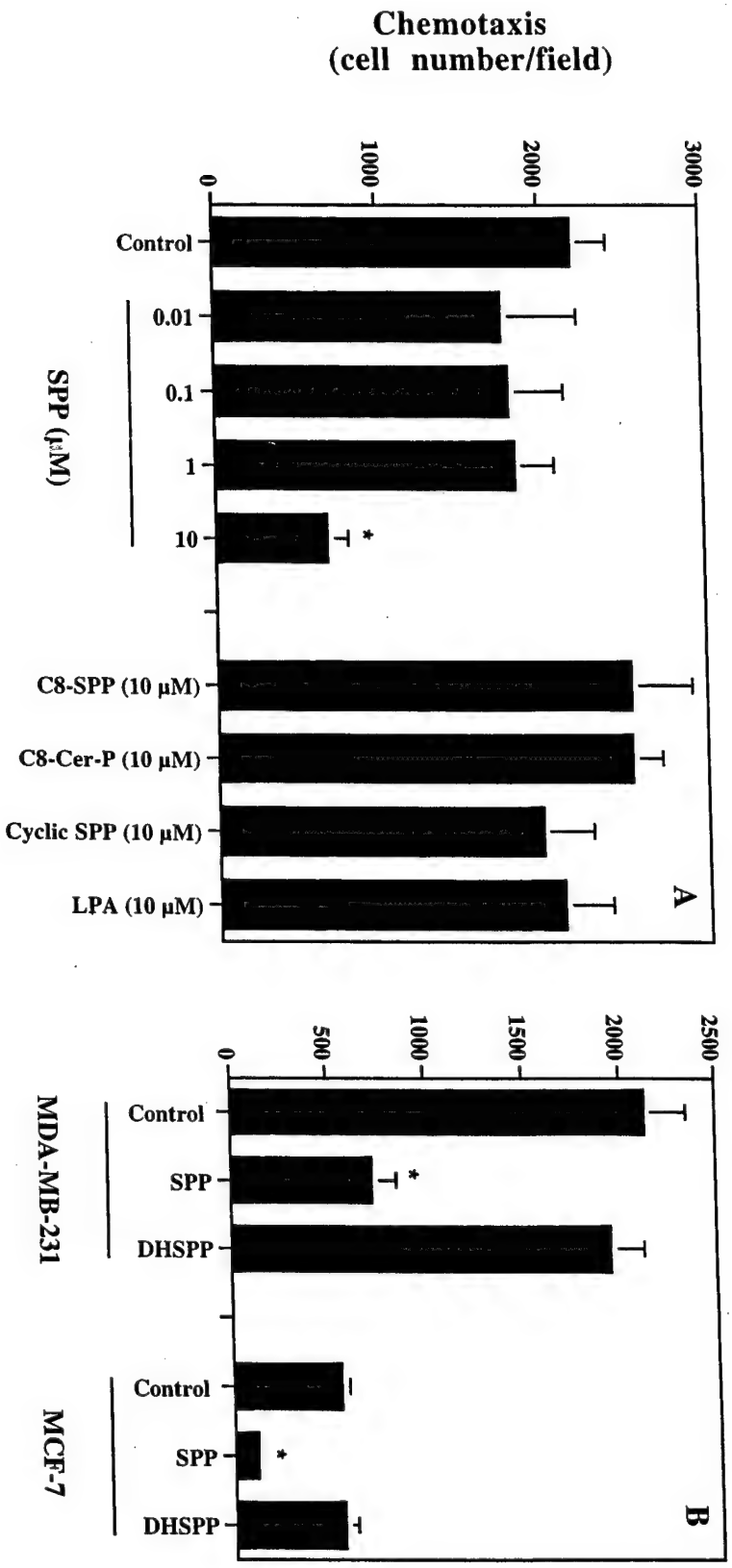

Chemotaxis
(cell number/field)
0
1000
2000
3000
Control
SPP (μM)
0.01
0.1
1
10
*
C8-SPP (10 μM)
C8-Cer-P (10 μM)
Cyclic SPP (10 μM)
LPA (10 μM)
A
0
500
1000
1500
2000
2500
MDA-MB-231
Control
SPP
*
DHSPP
MCF-7
Control
SPP
*
DHSPP
B

Fig 4

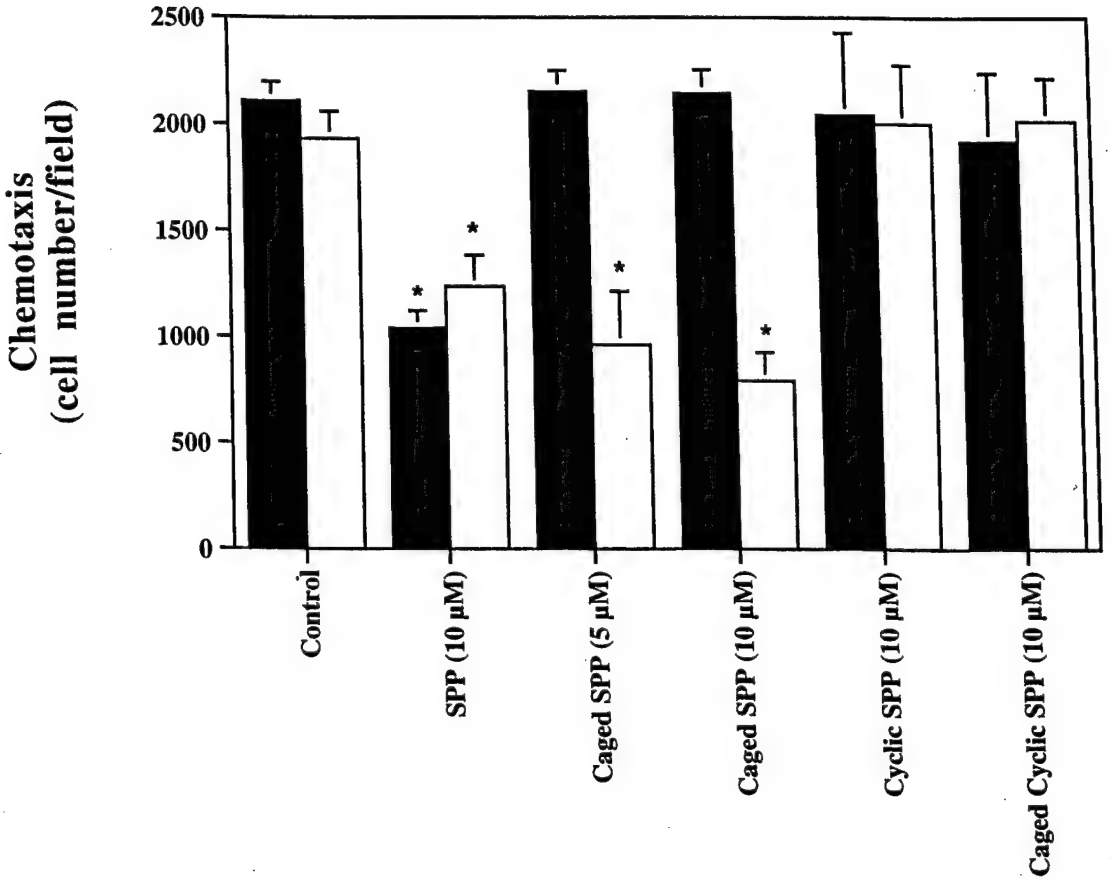
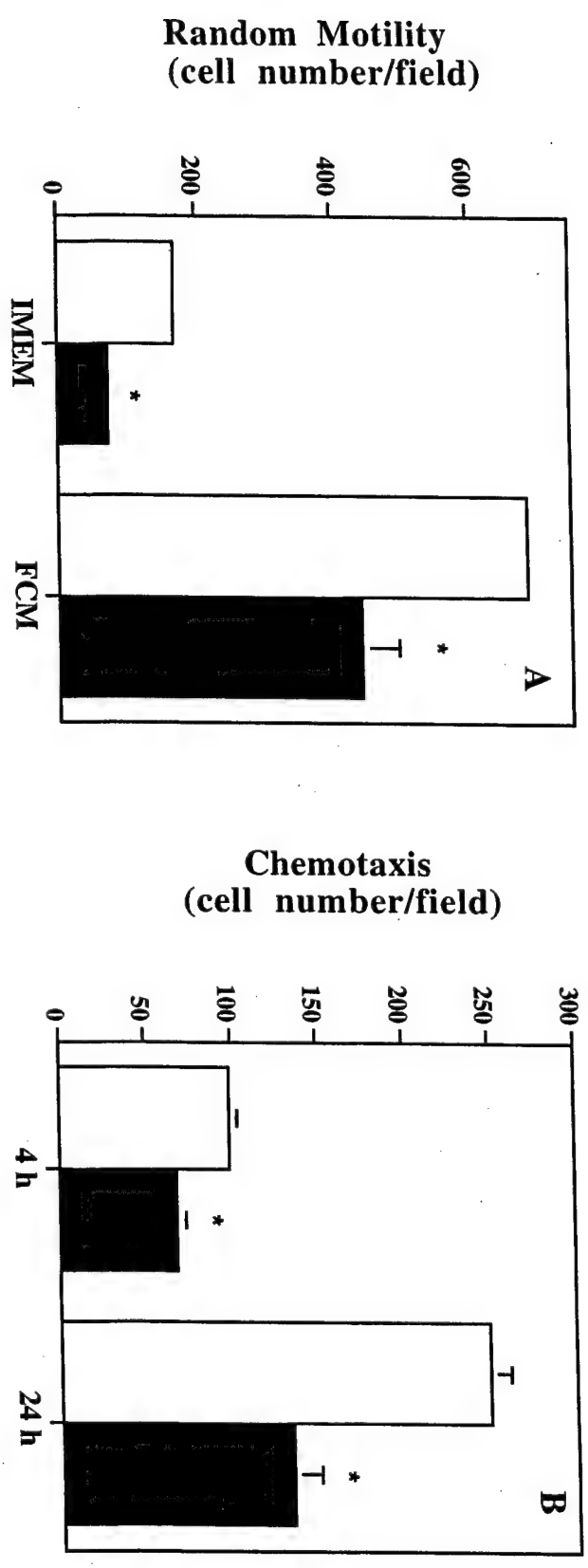

Chemotaxis
(cell number/field)
2500
2000
1500
1000
500
0
*
*
*
*
Control
SPP (10 µM)
Caged SPP (5 µM)
Caged SPP (10 µM)
Cyclic SPP (10 µM)
Caged Cyclic SPP (10 µM)

Fig 5


Random Motility
(cell number/field)
0
200
400
600
IMEM
FCM
*
*
T
A
Chemotaxis
(cell number/field)
0
50
100
150
200
250
300
4 h
24 h
–
–
*
T
T
*
B

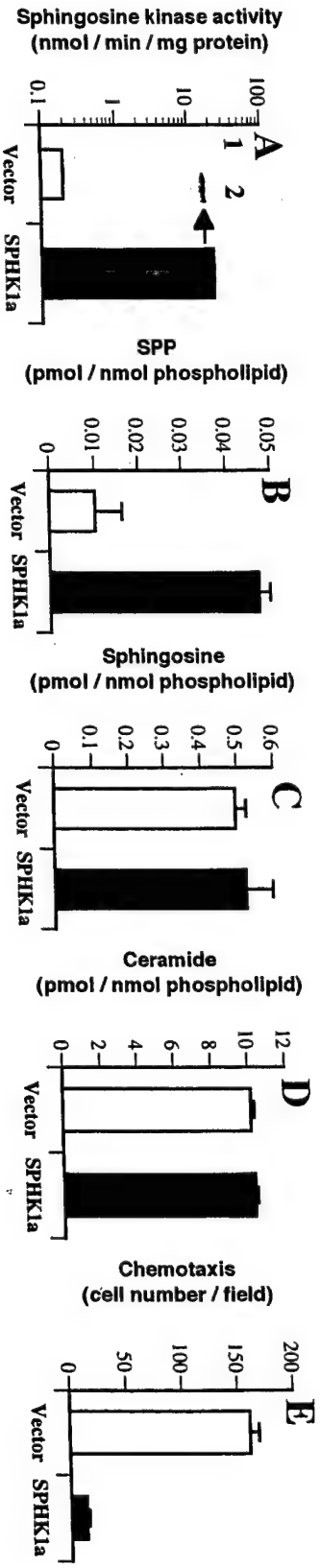

Sphingosine kinase activity
(nmol / min / mg protein)
0.1
1
10
100
A
1
2
Vector
SPHK1a
SPP
(pmol / nmol phospholipid)
0
0.01
0.02
0.03
0.04
0.05
B
Vector
SPHK1a
Sphingosine
(pmol / nmol phospholipid)
0
0.1
0.2
0.3
0.4
0.5
0.6
C
Vector
SPHK1a
Ceramide
(pmol / nmol phospholipid)
0
2
4
6
8
10
12
D
Vector
SPHK1a
Chemotaxis
(cell number / field)
0
50
100
150
200
E
Vector
SPHK1a

Fig 6

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Sphingosine 1-Phosphate Stimulates Cell Migration through a G_i-coupled Cell Surface Receptor

POTENTIAL INVOLVEMENT IN ANGIOGENESIS*

(Received for publication, April 22, 1999, and in revised form, August 20, 1999)

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Sphingosine 1-phosphate (SPP) has been implicated as a second messenger in cell proliferation and survival; however, some of its biological effects are due to binding to cell surface receptors. SPP has been shown to inhibit chemotaxis of a variety of cells, in some cases through intracellular actions, while in others through receptor-mediated effects. Surprisingly, we found that low concentrations of SPP (10–100 nM) increased chemotaxis of HEK293 cells overexpressing the G protein-coupled SPP receptor EDG-1. In agreement with previous findings in human breast cancer cells (Wang, F., Nohara, K., Olivera, O., Thompson, E. W., and Spiegel, S. (1999) *Exp. Cell Res.* 247, 17–28), SPP, at micromolar concentrations, inhibited chemotaxis of both vector- and EDG-1-overexpressing HEK293 cells. Nanomolar concentrations of SPP also induced a marked increase in chemotaxis of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC), which express the SPP receptors EDG-1 and EDG-3, while higher concentrations of SPP were less effective. Treatment with pertussis toxin, which ADP-ribosylates and inactivates G_i-coupled receptors, blocked SPP-induced chemotaxis. Checkerboard analysis indicated that SPP stimulates both chemotaxis and chemokinesis. Taken together, these data suggest that SPP stimulates cell migration by binding to EDG-1. Similar to SPP, sphinganine 1-phosphate (dihydro-SPP), which also binds to this family of SPP receptors, enhanced chemotaxis; whereas, another structurally related lysophospholipid, lysophosphatidic acid, did not compete with SPP for binding nor did it have significant effects on chemotaxis of endothelial cells. Furthermore, SPP increased proliferation of HUVEC and BAEC in a pertussis toxin-sensitive manner. SPP and dihydro-SPP also stimulated tube formation of BAEC grown on collagen gels (*in vitro* angiogenesis), and potentiated tube formation induced by basic fibroblast growth factor. Pertussis toxin treatment blocked SPP-, but not bFGF-stimulated *in vitro* angiogenesis. Our results suggest that SPP may play a role in angiogenesis through binding to endothelial cell G_i-coupled SPP receptors.

The sphingolipid metabolite sphingosine 1-phosphate (SPP)[1] is a bioactive lipid that regulates diverse biological effects and signaling pathways (reviewed in Ref. 1). SPP increases cell proliferation (2, 3) and opposes ceramide-mediated apoptosis (4–7) through an intracellular action (8, 9), yet some of its biological effects when added exogenously are due to binding to cell surface receptors. Pertussis toxin-sensitive G proteins are involved in some of the signaling pathways activated by SPP (10–15), suggesting that it activates a receptor coupled to a G_i/G_o protein. In agreement, low concentrations of SPP activate G_i protein-gated inward rectifying K^+ channels only when applied at the extracellular face of atrial myocytes (16).

Several reports have demonstrated that SPP inhibits cell motility. SPP inhibited chemotactic motility of mouse melanoma B16, mouse fibroblast BALB/3T3 clone A31, and several tumor cell lines at nanomolar concentrations (17–19). Moreover, SPP immobilized on glass beads markedly inhibited melanoma cell motility. However, pertussis toxin treatment did not block the effect of SPP, suggesting that in these cells SPP acts through a cell surface receptor, independently of pertussis toxin-sensitive G-proteins (20). In contrast, SPP inhibits chemotaxis of human breast cancer cells only at high (micromolar) concentrations, acting independently of EDG-1 (21).

We have recently identified SPP as a ligand for the G-protein-coupled receptor, endothelial differentiation gene-1 (EDG-1) (22). EDG-1 binds SPP with remarkable specificity and high affinity (K_D = 8 nM) (9, 22). Binding of SPP to EDG-1 resulted in inhibition of adenylate cyclase and activation of mitogen-activated protein kinase (both G_i-mediated), but did not mobilize calcium from internal stores (9, 23). In contrast, Okamoto *et al.* (12) found that in HEL cells overexpressing EDG-1, binding of SPP induced calcium mobilization (12).

Two other related G protein-coupled receptors, EDG-3 and EDG-5, have recently been shown to bind SPP with similar high affinity (14, 24), to confer responsiveness to SPP of a serum response element-driven reporter gene when expressed

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§ Supported by Postdoctoral Fellowship F32 GM19209 from the National Institutes of Health.

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[1] The abbreviations used are: SPP, sphingosine 1-phosphate; BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cells; GPCR, G protein-coupled receptors; dihydro-SPP, sphinganine 1-phosphate; LPA, lysophosphatidic acid; IMEM, Richter's improved minimal essential medium; bFGF, basic fibroblast growth factor; EDG-1, endothelial differentiation gene-1; SPC, sphingosylphosphorylcholine; RT-PCR, reverse transcriptase-polymerase chain reaction.

in Jurkat cells, and to allow SPP-stimulated $^{45}Ca^{2+}$ efflux in *Xenopus* oocytes (25). In agreement, overexpression of EDG-3 in Chinese hamster ovary cells led to phospholipase C activation and calcium mobilization induced by SPP, which was significantly inhibited by pertussis toxin (26). However, low concentrations of SPP mobilize calcium from internal sources in BAEC in a pertussis toxin-sensitive manner without activation of phospholipase C (27), suggesting the involvement of novel, unidentified signaling pathways in SPP-induced release of intracellular calcium.

Although the biological functions of the EDG family of GPCR are not completely understood, the EDG-1 transcript was originally cloned as an immediate-early gene induced during differentiation of HUVEC, cells of the vessel wall accessible to platelet-derived ligands, into capillary-like tubules (28). Moreover, SPP signaling in HEK293 cells overexpressing EDG-1 leads, by a Rho-dependent mechanism, to formation of a network of cell-cell aggregates resembling the network formation of differentiated endothelial cells and P-cadherin expression (22). Because SPP is stored and released from activated platelets and serum concentrations of SPP are estimated to be approximately 0.5 μM (29), about 60 times greater than the K_D for binding to EDG-1, we suggested that SPP might play an important role in angiogenesis acting through EDG-1 (22).

Angiogenesis, the process of new vessel formation from preexisting ones, or neovascularization, is a critical event for a variety of physiological processes, such as wound healing, embryonic development, corpus luteum formation, and menstruation. However, angiogenesis can be activated in response to tissue damage and is important in certain pathological conditions such as tumor growth and metastasis, rheumatoid arthritis, diabetic retinopathy, psoriasis, and cardiovascular diseases (30). Conversely, in states of inadequate tissue perfusion, such as myocardial or limb ischemia, enhanced angiogenesis is essential and beneficial (31). Endothelial cell migration and formation of new capillary tubes are required events in the angiogenic response. In this study, we investigated the potential role of SPP in angiogenesis by examining regulation of endothelial cell motility, proliferation, and tube formation through SPP receptors.

EXPERIMENTAL PROCEDURES

Materials—IMEM, penicillin/streptomycin, L-glutamine, amphotericin B, fetal calf serum, and fetal bovine serum were from Biofluids (Rockville, MD). Medium 199 was from Life Technologies (Gaithersburg, MD). Calf serum was from Colorado Serum Co. (Denver, CO), and Matrigel, bFGF, endothelial cell growth supplement, and rat tail type I collagen were from Collaborative Biomedical Products (Bedford, MA). SPP, dihydro-SPP, and sphingosylphosphorylcholine (SPC) were obtained from Biomol Research Laboratory Inc. (Plymouth Meeting, PA). 1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA) was from Avanti Polar Lipids, Inc. (Alabaster, AB). The DAB and Diff-Quik kits were from Sigma. [*methyl*-^{3}H]Thymidine (55 Ci/mmol) was purchased from Amersham Pharmacia Biotech.

Cell Culture—Human embryonic kidney cells (HEK293, ATCC CRL-1573) and HEK293-*EDG-1* cells, kindly provided by Drs. Menq-Jer Lee and Timothy Hla, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, with 1 mg/ml G418 sulfate for HEK293-*EDG-1*, as described previously (9, 22). BAEC were kindly provided by Dr. Luyuan Li and maintained in IMEM containing 10% fetal bovine serum supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 1 ng/ml bFGF. HUVECs were isolated as previously reported (32), and grown in medium 199 supplemented with gentamycin, 2 mM glutamine, 500 units/dl sodium heparin, 2.5 mg/dl amphotericin B, and 2 mg/dl endothelial cell growth supplement.

SPP Binding Assay—HUVEC or BAEC were washed with binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 15 mM sodium fluoride, 2 mM deoxypyridoxine, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin) and removed from dishes by scraping. Cells were then pelleted and resuspended in binding buffer containing 4 mg/ml BSA. 10^5 cells were incubated with 0.2 nM [^{32}P]SPP, synthesized enzymatically using recombinant sphingosine kinase (33) as described previously (9), in 0.2 ml of binding buffer plus 4 mg/ml BSA for 30 min at 4 °C in the absence or presence of 1000-fold excess unlabeled SPP or other lipid competitors, added as 4 mg/ml fatty acid-free BSA complexes. Cells were then pelleted at 8,000 rpm in a microcentrifuge, washed twice with binding buffer containing 0.4 mg/ml fatty acid-free BSA, resuspended in binding buffer without BSA and bound [^{32}P]SPP quantitated by scintillation counting. The phosphatase and protease inhibitors were included in the binding assays as a precaution against the possibility that cells which may have been damaged during scraping might leak phosphatases or proteases which could cleave SPP or Edg receptors, respectively. In addition, it has been proposed that cell surface lipid phosphatases which can cleave exogenous SPP exist (34). Nevertheless, identical specific binding of [^{32}P]SPP was obtained in the absence of the protease and phosphatase inhibitors. It should be pointed out that SPP is not metabolized during the binding assay. When [^{32}P]SPP was incubated in the absence or presence of endothelial cells under the same conditions as the binding assay, no decrease in the amount of [^{32}P]SPP was detected by TLC nor did any additional bands appear.

Reverse Transcriptase (RT)-PCR—The cDNA encoding the open reading frame of EDG-1 was amplified with the Gene Amp RNA-PCR kit (Perkin-Elmer) using RNA isolated with TRIzol Reagent (Life Technologies) and digested with RNase-free DNase I (RQ-1, Promega). The primers (Life Technologies) used for PCR amplification were 5′-GATATCATCGTCCGGCATTAC and 5′-ACCCTTCCCAGTGCATTGTTC for *EDG-1* (28); 5′-CACTCAGCAATGTACCTGTTCC and 5′-AACACCCAGTACGATGGTGAC for *EDG-5* (35, 36); and 5′-GACTGCTCTACCATCTTGCCC and 5′-GTAGATGACAGGGTTCATGGC for *EDG-3* (37). PCR reactions were performed for 30 cycles with denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 50 s. PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Migration—Chemotactic migration of cells in response to a gradient of SPP was measured in a modified Boyden chamber as described previously (21). In brief, polycarbonate filters (5 μm for BAEC and 8 μm for HUVEC) were coated with gelatin (0.1%) overnight. Cells were harvested by trypsinization, washed with serum-free IMEM containing 0.1% fatty acid-free BSA, and were added to the upper wells (24-multiwell Boyden microchambers) at 1×10^5 cells per well; the lower wells contained SPP diluted in serum-free IMEM containing 0.1% BSA. After 2 h at 37 °C in 5% CO_2, non-migratory cells on the upper membrane surface were removed with a cotton swab and the cells which traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik. The number of migratory cells per membrane was enumerated using a microscope with a ×20 objective. Each data point is the average number of cells in four random fields, each counted twice. Each determination represents the average ± S.D. of three individual wells. Checkerboard assays were carried out as described above except that various dilutions of SPP in fatty acid-free BSA were placed in the top and/or bottom wells of the Boyden chamber. In most of the experiments, unless indicated otherwise, cells were serum starved for 2 h prior to the assays.

[^{3}H]Thymidine Incorporation Assays—Cells were seeded at an initial density of 5×10^4 cells per well in 24-well plates and allowed to attach overnight. Confluent BAEC were growth arrested in culture media without bFGF for 48 h and then treated for 24 h with SPP or bFGF. Since the sensitivity of BAEC to stimulation by bFGF and SPP decreased with passage number, all experiments were carried out with cells at less than passage 12. Confluent HUVEC were serum-starved for 24 h and then treated with different concentration of SPP in medium 199 containing 1% FCS and 1000 units/dl heparin for 24 h. [^{3}H]Thymidine (1 μCi/ml) was added 8 h before termination of the assay, and [^{3}H]thymidine incorporation into DNA was measured as described (38). Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

In Vitro Angiogenesis—Three-dimensional collagen gel plates (24 well) were prepared by addition of 0.5 ml of a chilled solution of 0.7 mg/ml rat tail type I collagen in Dulbecco's modified Eagle's medium adjusted to neutral pH with $NaHCO_3$. After formation of the collagen gel (about 1–2 mm thickness), BAEC were seeded at 50,000 cells/well. At 80% confluency, culture medium was changed to media without bFGF and incubation was continued for 48 h, by which time the cells formed a monolayer on the gel. The cells were then treated with different concentrations of bFGF or SPP as indicated. Cultures were maintained at 37 °C for 48 h and then fixed with cold methanol. The gels were then soaked in phosphate-buffered saline/glycerol (1:1) and trans-

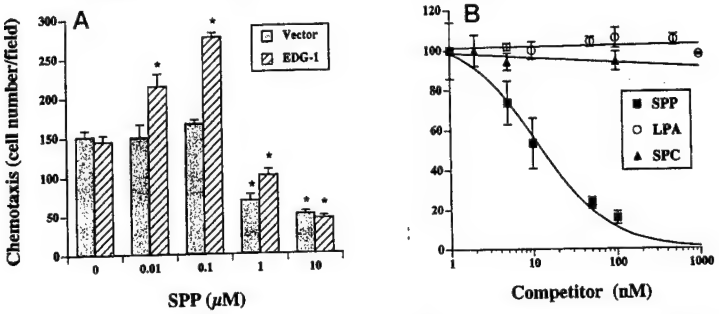

FIG. 1. **Effect of SPP on chemotaxis of HEK293 cells overexpressing EDG-1.** *A*, stable vector-transfected (*hatched bars*) or *EDG-1*-transfected (*closed bars*) HEK293 cells were treated with the indicated concentrations of SPP, and chemotaxis was measured as described inder "Experimental Procedures." Data are mean ± S.D. of triplicate determinations. Similar results were obtained in three independent experiments. The *asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$) compared with untreated cells. *B*, competition by lysopshingolipids for [^{32}P]SPP binding to HEK293 cells overexpressing EDG-1. HEK293-*EDG-1* cells were incubated with 0.2 nM [^{32}P]SPP in the absence or presence of the indicated concentrations of unlabeled SPP, LPA, or SPC and specific binding was measured as described under "Experimental Procedures."

ferred onto glass slides. The extent of tube-like structures that formed in the gel was measured as total length per field using computer-assisted imaging with a Hamamatsu C2400 video camera and a Zeiss Axioscope microscope to quantitate the extent of tube formation. Three culture wells were used for each sample, and three microscopic fields were examined for each well. Thus, each experimental point represents results from examination of nine microscopic fields.

RESULTS

Effect of SPP on Chemotaxis of EDG-1-overexpressing Cells—SPP was previously shown to inhibit chemotaxis of mouse melanoma cells by binding to a putative cell surface receptor (20). Since we have recently identified EDG-1 as a receptor for SPP (22), it was of interest to examine the involvement of EDG-1 in SPP-regulated cell motility. Human embryonic kidney 293 fibroblasts stably expressing EDG-1 (HEK293-*EDG-1*) were selected for these studies since they have high levels of specific SPP binding, whereas SPP binding is nearly undetectable to parental and vector transfected cells (9, 22). Surprisingly, low concentrations of SPP (10–100 nM) did not inhibit, but rather enhanced chemotaxis of HEK293-*EDG-1* cells by 1.5–1.9-fold, while chemotaxis of vector-transfected cells was not altered by these concentrations of SPP (Fig. 1*A*). The concentrations of SPP which stimulate chemotaxis of HEK293-*EDG-1* cells are in the same range as the measured affinity of EDG-1 for SPP (K_D = 8 nM) (22) and correlate closely with binding and inhibition of forskolin-stimulated cAMP accumulation in these cells (9). These results suggest that low concentrations of SPP may increase chemotaxis by binding to EDG-1. Higher concentration of SPP (1–10 μM), as previously reported in human breast cancer cells (21), inhibited, rather than stimulated, chemotaxis of both vector and *EDG-1*-transfected cells.

In contrast to undetectable levels of *EDG-1* mRNA in parental and vector transfected HEK293 cells (9, 39), HEK293-*EDG*-1 cells express very high levels of *EDG*-1 mRNA as detected by Northern analysis (9) and RT-PCR (Fig. 2*A*). Additionally, a low level of *EDG-3* mRNA and *EDG-5* mRNA were detected by RT-PCR in these cells (Fig. 2*A*). Similar to our previous results (9), both unlabeled SPP and dihydro-SPP effectively competed with [^{32}P]SPP for binding to EDG-1 (data not shown), whereas, LPA and SPC were completely ineffective (Fig. 1*B*). Moreover, LPA, even at concentrations as high as 10 μM, and for prolonged incubations, had no significant effect on SPP binding to HEK293-*EDG*-1 cells.

Expression of EDG Receptors in Endothelial Cells—Previously, it has been demonstrated that human endothelial cells express high levels of *EDG-1* mRNA (28). Thus, it was of interest to examine whether the other SPP receptors, EDG-3 and EDG-5 (24, 25, 40), are also expressed in HUVEC as well as BAEC which, based on indirect evidence, have been proposed to have putative G_i-coupled SPP receptors linked to calcium mobilization (27). Consistent with previous studies, RT-PCR analysis clearly demonstrated an *EDG-1* PCR amplification product of about 1300 base pairs, in agreement with the predicted size (1284 base pairs), in HUVEC and BAEC (Fig. 2, *B* and *C*). HUVEC and BAEC also apparently expressed somewhat lower levels of *EDG-3* and barely detectable *EDG-5* mRNA. It should be noted that the bovine SPP receptor cDNAs have not yet been cloned and sequenced; however, our PCR primers were designed to cover highly conserved regions. Restriction analysis of the HUVEC RT-PCR products yielded fragments of the expected sizes confirming their identity (data not shown). The entire open reading frame of each of the three SPP receptors is encoded within a single exon (40, 41), and RT-PCR primers which span an intron junction cannot be used to evaluate genomic DNA contamination. Therefore, controls without reverse transcriptase were performed in all cases (Fig. 2).

Effects of SPP on Chemotaxis of Endothelial Cells—Since SPP increased chemotaxis of HEK293 cells by apparently acting through EDG-1, the effect of SPP on chemotaxis of HUVEC and BAEC, which express EDG-1 and -3, was examined (Fig. 3, *A* and *B*). SPP stimulated chemotaxis of both HUVEC and BAEC, reaching a maximum effect at 1 μM (7- and 10-fold, respectively). Similar to the results with HEK293-*EDG-1* cells, concentrations of SPP greater than 1 μM were less effective, although significant enhancement of chemotaxis was still evident at higher concentrations. Interestingly, bFGF (20 ng/ml), a potent angiogenic factor (42), increased chemotaxis of BAEC to the same extent as did 1 μM SPP.

It has recently been shown that directional migration toward appropriate agonist ligands can be triggered via receptors coupled to G_i but not by agonists for receptors coupled to two other G proteins, G_s and G_q (43). Because biochemical evidence and the yeast two-hybrid system indicate that EDG-1 is capable of interaction with $G\alpha_{i1}$ and $G\alpha_{i3}$ (44), we investigated the possibility that G_i proteins may be involved in the chemotactic response induced by SPP. To this end, HUVEC were treated with pertussis toxin, which ADP-ribosylates and inactivates G_i and G_o proteins, prior to addition of SPP. Pertussis toxin pretreatment completely abolished SPP-induced chemotaxis (Fig. 3*C*).

SPP Stimulates Directional Migration—We next determined

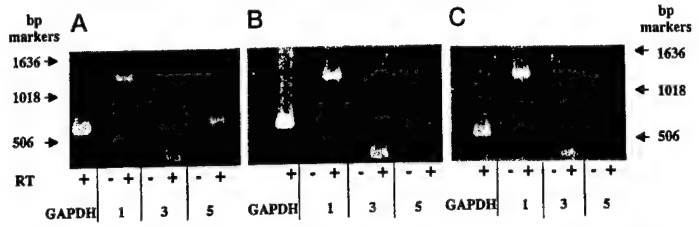

FIG. 2. **Expression of EDG receptor mRNAs in endothelial cells.** RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *EDG-1*, *EDG-3*, and *EDG-5* expression was carried out with RNA isolated from HEK293-*EDG-1* cells (*A*), HUVEC (*B*), or BAEC (*C*) as described under "Experimental Procedures" in the absence (−) or presence (+) of MULV-RT (RT). Similar results were found in two independent experiments. *bp*, base pair(s).

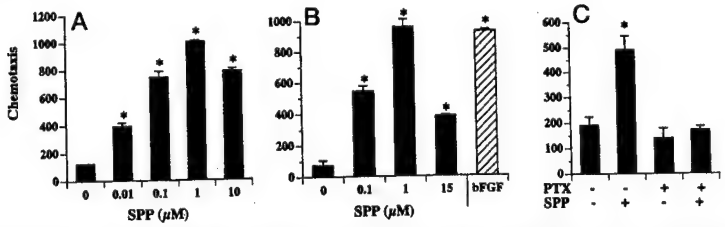

FIG. 3. **Effect of SPP on chemotaxis of HUVEC and BAEC.** *A*, HUVEC were allowed to migrate toward the indicated concentrations of SPP and chemotaxis was measured as described under "Experimental Procedures." *B*, BAEC were allowed to migrate toward different concentrations of SPP or 20 ng/ml bFGF as indicated, and chemotaxis was measured. Data are mean ± S.D. of triplicate determinations. Similar results were obtained in at least three independent experiments. *C*, HUVEC were pretreated with vehicle or 200 ng/ml pertussis toxin for 3 h, and then allowed to migrate toward vehicle or 100 nM SPP as indicated. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$).

whether the effect of SPP was mediated by enhanced directed migration in response to the gradient of chemoattractant (chemotaxis) or by increased random motility due to the presence of the chemoattractant itself (chemokinesis). Checkerboard assays were performed with various concentration of SPP in the top, bottom, or both chambers of the Boyden apparatus. The greatest numbers of cells were found to migrate either along the chemotactic gradient, *i.e.* toward increasing concentrations of SPP in the bottom chamber (Table I, *bold*), and also in the direction of the increasing chemokinetic gradient, *i.e.* when the concentration of SPP was the same in both the top and bottom chambers (Table I, *italic*), indicating that SPP stimulates both chemokinetic and chemotactic responses.

Binding of SPP and Dihydro-SPP Correlates with Stimulation of Cell Migration—It was important to determine whether [^{32}P]SPP was specifically bound to the endogenous SPP receptors on HUVEC. As shown in Fig. 4*A*, in HUVEC there was significant specific binding of both SPP and sphinganine 1-phosphate (dihydro-SPP), which lacks the double bond at the 4-position. Moreover, dihydro-SPP also markedly enhanced chemotaxis (Fig. 4*B*). The structure of SPP is similar to that of LPA, another serum-borne lysolipid that binds and signals through the related G_i-coupled receptors, EDG-2 and EDG-4 (45–47). However, excess LPA did not compete with [^{32}P]SPP for binding to HUVEC, nor did it have a significant stimulatory effect on chemotaxis. SPC had no significant effect on specific [^{32}P]SPP binding and had a small but not statistically significant effect on chemotaxis (Fig. 4, *A* and *B*). Similar results were obtained with BAEC, where only SPP and dihydro-SPP, albeit less potently, competed with labeled SPP for binding, whereas LPA and SPC had no significant effect. These results are consistent with our previous observation that dihydro-SPP blocked binding to HEK293 cells overexpressing EDG-1 in a dose-dependent manner similar to unlabeled SPP (9, 24), while neither LPA nor SPC had a significant effect on SPP binding (Fig. 1*B*). It should be pointed out that in these studies, all lysosphingolipids were added to cells as 0.4% BSA complexes, using conditions which were found to be optimal for binding of SPP to its receptor (9, 24), whereas these might not be optimal conditions for binding of LPA to EDG-2 and EDG-4. For example, LPA prepared as a 1% BSA complex promoted survival of Schwann cells, while SPP as a 0.01% complex was ineffective (48). We also examined the binding affinity of SPP for its putative receptor on endothelial cells by displacing bound [^{32}P]SPP with increasing concentrations of unlabeled SPP. 50% of the bound [^{32}P]SPP was competed at 10 nM unlabeled SPP in BAEC. Thus, binding of SPP to endothelial cells is also of high affinity and in agreement with the K_d of EDG-1 (8.6 nM). These results indicate that there is an excellent correlation between the K_d and the concentration-dependent effect of SPP on cell migration.

TABLE I
Checkerboard analyses of HUVEC migration induced by SPP

The checkerboard assay was arranged with increasing concentrations of SPP in the lower chamber (bold indicate migration due to chemoattaction) or increasing concentrations of SPP in both upper and lower chambers (italics indicate migration due to chemokinesis) and cell migration assays were performed as described under Materials and Methods. Data are mean ± S.D. of triplicate determinations.

Lower chamber	Upper chamber			
	0	10	100	1000
nM				
0	●●● ± ●●●	147 ± 24	177 ± 54	298 ± 14
10	●●● ± ●●●	*347 ± 24*	317 ± 53	390 ± 32
100	●●● ± ●●●	677 ± 18	***616 ± 108***	506 ± 7
1000	●●● ± ●●●	1001 ± 17	904 ± 101	*823 ± 24*

SPP Stimulates Proliferation of HUVEC and BAEC—Many angiogenic factors, in addition to enhancing chemotaxis, stimulate *in vitro* proliferation of endothelial cells (49–51). Since SPP increased chemotaxis of endothelial cells, and has previously been shown to be a potent mitogen for diverse cell types (1, 2, 52), it was of interest to examine the effects of SPP on proliferation of endothelial cells. SPP treatment of HUVEC induced a dose-dependent increase of DNA synthesis as meas-

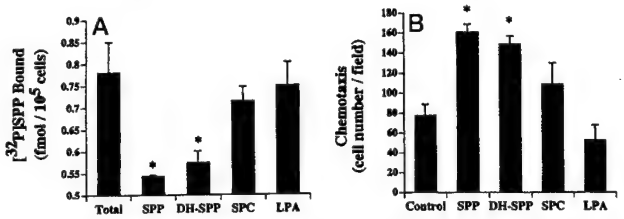

FIG. 4. **SPP and dihydro-SPP, but not LPA, specifically compete with [^{32}P]SPP binding and stimulate chemotaxis in HUVEC.** *A*, effects of SPP and other lysophospholipids on binding of [^{32}P]SPP to HUVEC. HUVEC were incubated in the presence of 0.2 nM [^{32}P]SPP for 30 min at 4 °C in the absence or presence of 300 nM unlabeled SPP, dihydro-SPP (DH-SPP), SPC, or LPA and binding was measured as described under "Experimental Procedures." *B*, HUVEC were allowed to migrate toward the indicated lysophospholipids (100 nM) and chemotaxis was measured. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$).

F5 ured by [^{3}H]thymidine incorporation with a maximum effect at 0.1–1 μM (Fig. 5*A*). Similar to SPP, 1 μM dihydro-SPP also stimulated DNA synthesis in HUVEC by 1.83 ± 0.1-fold, whereas LPA at a concentration as high as 10 μM had no significant effect. In agreement with our previous reports in various cell types (53–55), 10 μM SPC stimulated DNA synthesis by 1.95 ± 0.1-fold.

SPP was also mitogenic for BAEC; however, a maximal effect in these cells required higher concentrations (1–10 μM). bFGF has been reported to be a potent endothelial cell mitogen (56). Surprisingly, although bFGF stimulated proliferation of BAEC, at optimal concentrations it was only as effective as 1 μM SPP and less effective than 10 μM SPP (Fig. 5*B*). It should be noted that sensitivity of BAEC to SPP decreased with increasing passage number, similar to a previous report on the effect of passage number on bFGF responses (57), varied with passage number. In addition to SPP, dihydro-SPP stimulated DNA synthesis, whereas we found that LPA at a concentration up to 10 μM was not mitogenic, in fair agreement with previous studies where LPA only stimulated DNA synthesis in BAEC at concentrations around 30 μM (57).

To investigate the possibility that a G_i-coupled receptor may be involved in the proliferative response induced by SPP, endothelial cells were treated with pertussis toxin prior to addition of SPP. Both HUVEC and BAEC are more sensitive to pertussis toxin than Swiss 3T3 fibroblasts. In contrast with our previous studies with quiescent Swiss 3T3 fibroblasts (10, 11), where half of the stimulated DNA synthesis was still evident even at the highest effective concentration of pertussis toxin, pertussis toxin pretreatment of HUVEC and BAEC completely inhibited the SPP-induced mitogenic response, while it had no significant effect on DNA synthesis induced by bFGF (Fig. 5*C*).

SPP Induces Capillary-like Tube Formation in Vitro—Later stages of angiogenesis require morphological alterations of endothelial cells, which result in lumen formation (31). Critical steps in angiogenesis, such as migration and differentiation, have been studied using an *in vitro* model of angiogenesis in which cultured endothelial cells are induced to invade a three-dimensional collagen gel where they form a network of capillary-like structures or tubes when stimulated by angiogenic factors (58, 59). This phenomenon is thought to mimic the formation of new blood vessels *in vivo*. In agreement with previous studies (59), confluent monolayers of BAEC treated with bFGF (50 ng/ml) formed networks of capillary-like tubular
F6 structures within the gel (Fig. 6*A*). In contrast, little invasion or network cord formation and only a few short capillary sprouts originating from untreated BAEC embedded in collagen gels were detected. SPP evoked a dose-dependent increase in the formation of capillary-like tubes of BAEC invading the collagen gel. Apparently thinner tubes were formed in response to lower doses of SPP than those formed in response to bFGF.

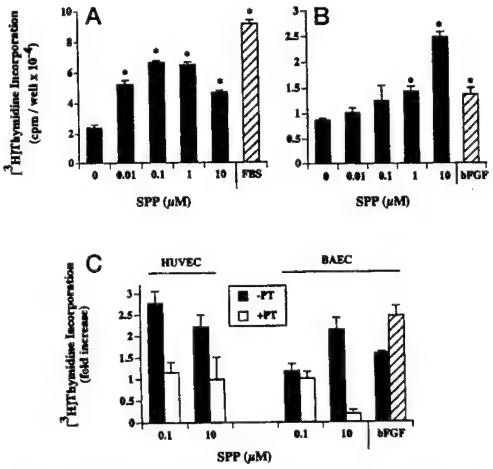

FIG. 5. **The effect of SPP on DNA synthesis of HUVEC and BAEC.** Quiescent HUVEC (*A*) or BAEC (*B*) were treated with the indicated concentrations of SPP or 20 ng/ml bFGF for 24 h and DNA synthesis as measured by [^{3}H]thymidine incorporation was determined as described under "Experimental Procedures." Data are mean ± S.D. of triplicate determinations and are representative of at least three independent experiments. *Asterisks* indicate statistical significance determined by Student's *t* test ($p \leq 0.01$). *C*, quiescent HUVEC and BAEC were incubated in the absence (*filled bars*) or presence of 20 ng/ml pertussis toxin (*open* or *hatched bars*). After 2 h, cells were washed and exposed to the indicated concentrations of SPP or bFGF for 24 h and [^{3}H]thymidine incorporation was measured. Data are expressed as fold increases compared with non-stimulated cells.

Quantitative evaluation of tube formation revealed that SPP, similar to bFGF, markedly increased the length of the endothelial tubular structures (Fig. 6*B*). There was an additive effect when SPP was applied together with bFGF, suggesting that SPP can potentiate the effect of bFGF on *in vitro* angiogenesis. Similar to SPP, dihydro-SPP, also markedly enhanced capillary-like tube formation of BAEC, whereas LPA was completely inactive and SPC had a small but significant effect (Fig. 6*C*). Pertussis toxin not only inhibited endothelial cell migration and proliferation induced by SPP, it also markedly decreased the SPP-induced tube formation (Fig. 6*C*). In sharp contrast, pertussis toxin had no effect on tube formation induced by bFGF.

DISCUSSION

Previously, many studies have shown that SPP inhibits chemotaxis of diverse cell types (17, 19, 20, 60, 61). Although in

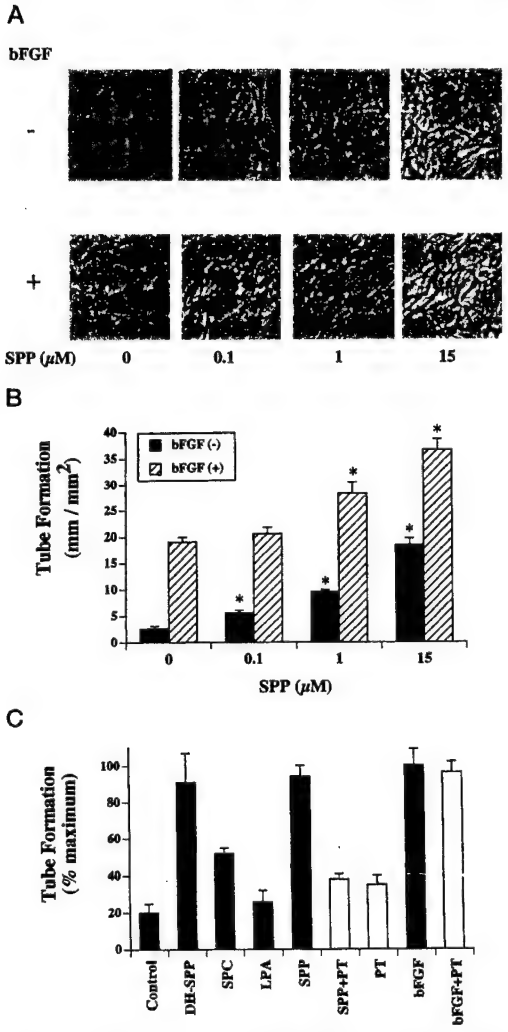

FIG. 6. **Induction of *in vitro* angiogenesis in collagen gels by SPP is attenuated by pertussis toxin.** BAEC were treated with the indicated concentration of SPP in the absence or presence of bFGF (50 ng/ml) and capillary tube formation on collagen gels was examined as described under "Experimental Procedures." *A*, representative phase-contrast micrographs of BAEC after 4 days of incubation in normal medium (*upper panels*) or in the presence of 50 ng/ml bFGF (*lower panels*) and the indicated concentrations of SPP. *B*, quantitative analysis of capillary tube formation. Data are expressed as length of tubes per square millimeter (n = 4 pairs of duplicates). *Asterisks* indicate statistical significance determined by Student's t test ($p \leq 0.01$). *C*, BAEC were treated in the absence or presence of pertussis toxin (*PT*, 20 ng/ml), without or with the indicated lysophospholipids (1 μM) or bFGF (50 ng/ml) and capillary tube formation on collagen gels was determined as described under "Experimental Procedures." Data are expressed relative to the maximum response elicited by bFGF.

human breast cancer cells, SPP inhibited chemotaxis independently of EDG-1 (21), a wealth of evidence suggests that in many other cell lines, SPP inhibits chemotaxis through unidentified cell surface receptors (17, 20, 62). Unexpectedly, we found in this study that binding of SPP to its G_i protein-coupled receptor EDG-1 markedly increased cell motility. In accord with its affinity for EDG-1, SPP at nanomolar concentrations increased chemotaxis of EDG-1-transfected but not vector-transfected HEK293 cells, as well as HUVEC and BAEC which constitutively express EDG-1.

Although a recent study demonstrated that LPA can bind to EDG-1 leading to receptor phosphorylation, ERK activation, as well as Rho-dependent morphogenesis and P-cadherin expression (39), in our study, LPA, even at a concentration as high as 10 μM, did not compete for binding of radiolabeled SPP to HEK293-*EDG-1* cells. In agreement, LPA did not displace bound [^{32}P]SPP from endothelial cells nor did it stimulate chemotaxis of HUVEC. In contrast, dihydro-SPP, which binds to EDG-1 (9), EDG-3, and EDG-5 (24), was as potent as SPP in induction of chemotaxis. However, LPA, similarly to SPP, can mimic serum in inducing invasion of carcinoma and hepatoma cells into monolayers of mesothelial cells (63). Although the underlying mechanism of this effect is not clear, it may involve increased cell adhesion rather than enhanced cell motility. Moreover, in most other cell types, LPA stimulates chemokinesis and chemotaxis (62, 64–66), which might be due to binding to its specific receptors, EDG-2 and EDG-4 (45–48).

We have previously shown that the SPP-induced cAMP decrease (9) and ERK2 activation in EDG-1-transfected cells (22) were completely blocked by pretreatment with pertussis toxin, which uncouples G_i from GPCR. Similarly, preincubation with pertussis toxin abolished the effect of SPP on migration of endothelial cells. Collectively, these findings suggest that binding of SPP to the serpentine receptor EDG-1 on the endothelial cell surface activates a pertussis toxin-sensitive G_i protein crucial for chemotaxis. In agreement, it has recently been demonstrated that activation of $G\alpha_i$-coupled receptors and the subsequent release of $G_{\beta\gamma}$ dimers is required to initiate signal transduction leading to directed cell migration (43, 67). It should be noted that the receptors for all known leukocyte chemoattractants, including the chemokines, are members of a seven-transmembrane domain superfamily and coupled to a variety of G_α subunits (68, 69). Moreover, pertussis toxin inhibits chemotaxis by preventing chemoattractant receptors from activating trimeric G proteins of the G_i subfamily (67).

Endothelial cells are accessible to platelet-derived ligands in the serum, and might be the target of serum-borne SPP which regulates their proliferation, migration, and differentiation into capillary-like tubules, important aspects of angiogenesis (31). SPP is well established as a potent mitogen for diverse cell types (reviewed in Ref. 1). Indeed, we found that SPP also stimulated DNA synthesis in endothelial cells and this was completely blocked by pertussis toxin, suggesting a role for G_i in this process. In agreement, SPP was recently reported to stimulate HUVEC proliferation (70), albeit at somewhat higher concentrations which resembled the dose-response curve that we found for BAEC. It is possible that differences in sensitivity to SPP might arise from differences in passage numbers as has been shown for responses to bFGF (57). Interestingly, SPP stimulated DNA synthesis in Swiss 3T3 fibroblasts only at high concentrations and in contrast to endothelial cells, was only partially inhibited by pertussis toxin (10). Moreover, in these cells, DNA synthesis was significantly and specifically increased by microinjection of SPP (9). Pertussis toxin reduced the level of DNA synthesis caused by exogenous SPP to approximately the same level as that induced by microinjected SPP (9). Thus it is likely that in fibroblasts, both intracellular as well as receptor-mediated responses to SPP are involved in its mitogenic effect. It is possible that there is a complex interplay between cell surface receptor signaling and intracellular targets for SPP, which can contribute to its mitogenic response in

certain cell types. Thus, SPP, may act in a similar manner to other bioactive lipids, such as leukotriene B4 (LTB4), which act through cell surface receptors and also have intracellular targets. LTB4 is a potent chemoattractant that is primarily involved in activation of inflammatory cells by binding to its GPCR. However, it can also bind and activate the intranuclear transcription factor PPARα, resulting in the activation of genes that terminate inflammatory processes (71).

In this study, we also observed that SPP in addition to stimulating both random, nondirectional migration (chemokinesis) and directional migration (chemotaxis) of endothelial cells, it also markedly enhanced morphogenetic differentiation of endothelial cells *in vitro*. BAEC cultured on collagen showed increased tube formation in the presence of SPP, demonstrating that SPP stimulates endothelial cell differentiation as well as migration. Moreover, pertussis toxin not only inhibited endothelial cell migration and proliferation induced by SPP, it also completely inhibited SPP-induced, but not bFGF-induced, formation of capillary-like tubes. Taken together, these results demonstrate that SPP has angiogenic activity *in vitro* acting through a G_i-coupled cell surface receptor.

In contrast to its effect on HEK293-*EDG*-1 cells, SPP did not stimulate chemotaxis (Fig. 1), proliferation (9), or morphogenetic differentiation in vector-transfected HEK293 cells (22), further supporting the notion that the effects that we observed on chemotaxis, proliferation, and tube formation of endothelial cells are mediated by EDG-1. In contrast, parental and vector-transfected HEK293 cells do show activation of ERK mitogen-activated protein kinases in response to SPP which might be attributable to endogenous EDG-3 or EDG-5. Taken together with our results, these findings suggest that EDG-1 may mediate these stimulatory effects of SPP on endothelial cells. However, as the other known SPP receptor EDG-3, which is also expressed in HUVEC, has been shown in some cases to couple to pertussis toxin-sensitive G proteins as well as to Rho and phospholipase C signaling pathways (14, 26), this receptor might also contribute to the effects of SPP on tube formation. Recently, the polycyclic anionic compound suramin has been shown to selectively antagonize SPP-activated calcium transients in EDG-3, but not in EDG-1 or EDG-5 expressing oocytes, with an IC_{50} of ~22 μM, suggesting that it is an antagonist selective for the EDG-3 GPCR isotype (72). However, addition of 100 μM suramin did not abrogate the ability of SPP to induce tube formation in BAEC,[2] suggesting that this effect is mediated by binding of SPP to EDG-1, the major endogenous SPP receptor on endothelial cells, rather than EDG-3. In contrast, suramin inhibited Rho-dependent neurite retraction induced by SPP in N1E-115 neuronal cells (73) and SPP-induced invasion of T-lymphoma cells (74). However, in agreement with its lack of effect on SPP-induced tube formation, suramin had no significant effect on proliferation, stress fiber formation, and FAK phosphorylation induced by SPP in Swiss 3T3 fibroblasts (75).

Similar to SPP, dihydro-SPP also markedly enhanced cell migration and capillary-like tube formation of BAEC, whereas LPA was completely inactive. Another structurally related analog of SPP, SPC, although it had no significant effect on binding of labeled SPP to endothelial cells, it had a small but significant stimulatory effect on tube formation, and at high concentrations, it also stimulated proliferation. These effects might be related to the potent action of SPC as a wound healing agent (55). Interestingly, high micromolar concentrations of SPC activated calcium transients in EDG-1, -3, and -5 expressing oocytes (72) and SRE-driven gene transcription in Jurkat T cells (25). While these observations suggest that SPC might be a very low affinity ligand for these EDG receptors, it is also possible that these effects were not mediated by SPC itself as it was recently found that commercial preparations of SPC are contaminated with highly potent alkenyl glycero-3-phosphates (76).

Some of the downstream effects of SPP signaling through EDG-1, such as decreased cAMP (9, 23) and activation of ERK2 (22), are pertussis toxin-sensitive, while others, such as morphogenetic differentiation, are pertussis toxin-insensitive but inhibited by the C3 exoenzyme (22) which blocks signaling through the small GTPase Rho (77). Thus it appears that EDG-1 can couple to G_i proteins as previously reported (44), as well as to $G_{12/13}$ proteins which are thought to regulate Rho (78, 79), and thus might also be important for SPP-induced chemotaxis and angiogenesis. Recently, it has been demonstrated that disruption of the gene encoding $G\alpha_{13}$ impaired the ability of endothelial cells to organize into a vascular system and greatly impaired migratory responses (80), suggesting that in addition to G_i, other proteins including $G\alpha_{13}$, might be required for regulation of cell movement. In agreement, T-lymphoma cell invasion is dependent on SPP receptor-mediated RhoA and phospholipase C signaling pathways which lead to pseudopodia formation (74).

In summary, in this study we have demonstrated that SPP has appropriate properties to be considered as a *bona fide* angiogenic factor, *i.e.* it stimulates chemokinetic and chemotactic motility, proliferation of vascular endothelial cells, and stimulates angiogenesis *in vitro*, similarly to the known angiogenic factor bFGF. Because bFGF and SPP have an additive effect on formation of capillary-like tubes by endothelial cells invading collagen gels, SPP may be a specific type of angiogenic factor. It is possible that SPP plays a role in normal blood vessel formation or in injury, when local production of SPP could be increased by activated platelets, and extravasation of intravascular fluid could also present SPP into tissues at concentrations sufficient to promote angiogenesis and wound healing. Elucidation of the molecular mechanisms by which SPP stimulates cell migration and angiogenesis might provide clues for development of new therapeutic agents to either promote or block these processes by targeting the EDG family of GPCR.

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Report

Glycosphingolipid composition of MDA-MB-231 and MCF-7 human breast cancer cell lines

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Key words: gangliosides, neutral glycolipids, GM3, breast cancer cell line

Summary

Much evidence has shown that glycosphingolipids are involved in cellular recognition, regulation of cell growth, and metastasis. In the present study, the major glycosphingolipids of two widely studied human breast cancer cell lines were examined. The MCF-7 cell line has functional estrogen and EGF receptors, is dependent on estrogen and EGF for growth, and is uninvasive, while MDA-MB-231 cells are a model for more aggressive, hormone-independent breast cancer. There was twice as much neutral glycolipid in MCF-7 cells as in MDA-MB-231 cells. The major neutral glycolipids in MDA-MB-231 cells were identified as CTH and globoside. MCF-7 cells also contained as the major neutral glycolipids CTH as well as globoside and two other glycolipids which were tentatively identified as galactosylgloboside and fucosylgalactosylgloboside by exoglycosidase treatments. Conversely, the ganglioside content was four fold higher in MDA-MB-231 cells compared to MCF-7 cells. The abundant gangliosides in both cell lines were GM3, GM2, GM1, and GD1a. A minor monosialoganglioside was detected in MDA-MB-231 cells. The striking 18 fold greater amount of GM3 in MDA-MB-231 cells may have important implications because GM3 has been suggested to be involved in regulation of growth factor functions. In agreement, insertion of ganglioside GM3 into the plasma membrane of MCF-7 cells blocked the growth stimulatory effect of EGF.

Abbreviations: CTB – B subunit of cholera toxin; CMH – monohexosylceramide; CDH – lactosylceramide; CTH or Gb_3Cer – Galα1-4Galβ1-4Glcβ1-1′Cer; Cer – globoside or Cb_4Cer – GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer; EGF – epidermal growth factor; Forssman glycolipid – $IV^3GalNAc\alpha\text{-}Gb_4Cer$; asialo-GM1 – Galβ1-3GalNAcβ1-4Galβ1-β-4Glcβ1-1′Cer; paragloboside or nLc_4Cer – Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; nLc_6Cer – Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; NeuAc – N-acetylneuraminic acid; TLC – thin-layer chromatography

Introduction

Glycosphingolipids and gangliosides, the sialylated glycosphingolipids, have long been implicated in cellular proliferation, cell-cell interactions, differentiation, and modulation of receptor functions [1–11]. We demonstrated that ganglioside GM1 is involved in regulation of cell growth by regulating calcium influx in a protein kinase C-independent manner using the B subunit of cholera toxin, which

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binds with very high affinity to ganglioside GM1 [2]. Similarly, it has also been demonstrated that ganglioside GM1 triggers a sustained calcium response through a $p56^{lck}$-dependent pathway in human Jurkat T cells [3]. Recent studies have shown the association of gangliosides with other membrane proteins which can convey signals to cytosolic components [4, 5]. Ganglioside GM3 modulates tyrosine phosphorylation of the epidermal growth factor (EGF) receptor [7] by a specific association with the receptor [8] which inhibits receptor dimerization and consequent tyrosine kinase activity [8]. In addition, in human glioma U-242MG and Swiss 3T3 cells, complex gangliosides inhibit platelet-derived growth factor-stimulated cellular proliferation by blocking receptor dimerization [8]. Complexes of alpha-galactosyl derivatives of GD1b with several proteins, including $p53/56^{lyn}$, a serine kinase, and the high affinity IgE receptor, were observed in rat basophilic leukemia cells, and it has been suggested that such complexes may play an important role in receptor-mediated signal transduction [5].

Oncogenic transformation is accompanied by drastic changes in glycosphingolipid composition [1]. It has been shown that relatively large amounts of gangliosides are shed by malignant cells and that these gangliosides may be immunosuppressive molecules inhibiting T cell responses [9]. Tumor gangliosides also specifically modulate cellular immune responses *in vivo*, which may explain the enhancement of tumor formation by these molecules [10]. Gangliosides have also been implicated in metastasis, and recently GM3 has been shown to be involved in integrin receptor function and adhesion to the extracellular matrix, one of the critical elements in cancer cell invasion [11].

Cell lines established from human breast cancer provide *in vitro* models for breast cancer in various stages of progression. Onset and progression of human breast cancer is dependent upon ovarian estrogens [12]. The MCF-7 cell line has functional estrogen receptors (ER) and is dependent on estrogen for growth *in vitro* and in the nude mouse [12, 13]. These cells also express EGF receptors and their growth is stimulated by EGF. Other human breast cancer cell lines which lack ER, such as MDA-MB-231, are unresponsive to estrogen and anti-estrogens (tamoxifen and benzothiophene), and provide models for more aggressive, hormone-independent breast cancers. Although MDA-MB-231 cells overexpress EGF receptors, they are not responsive to EGF. The presence (ER^+) or lack (ER^-) of estrogen receptors in human breast cancer cells is an important determinant of both prognosis and choice of treatment, a poorer prognosis associating with the ER^- phenotype while positive ER status predicts a longer disease-free survival and overall survival independent of axillary lymph node status [12, 13]. Recently, the presence of the intermediate filament glycoprotein vimentin has also been found to have prognostic significance equivalent to that afforded by ER [14], but the correlation between vimentin expression and prognosis of breast cancer is still a controversial issue. In this regard, biosynthesis of glycosphingolipids (especially lactosylceramide, globotriosylceramide, and ganglioside GM3) is markedly reduced in the absence of a vimentin intermediate filament network [15].

A few minor neutral glycolipids were previously characterized in MCF-7 cells during identification of antigens detected by monoclonal antibodies which react with cancer cells [16, 17]. However, the major glycosphingolipid compositions of human breast cancer cell lines have not been examined previously. In the present study, we examined the major glycosphingolipids of MDA-MB-231 and MCF-7 cells to explore the possible linkage between their composition and the progression of breast cancer, and found a striking quantitative difference in the ganglioside composition between these two cell lines.

Materials and methods

Standard glycolipids

The following neutral glycolipids and gangliosides were purchased from Sigma: CMH, CDH, CTH, globoside, asialo-GM1, GM3, GM2, and GM1. Two sialylparaglobosides, $IV^3NeuAc\alpha$-nLc_4Cer and $IV^6NeuAc\alpha$-nLc_4Cer, were from Wako Pure Chemical Industries. Paragloboside and nLc_6Cer was prepared from $IV^3NeuAc\alpha$-nLc_4Cer and

VI^6NeuGcα-nLc_6Cer (kindly provided by Dr. Basu, University of Notre Dame) by sialidase hydrolysis as described below.

Cell lines

Human breast cancer cell lines MDA-MB-231 and MCF-7 cells were obtained from the Lombardi Cancer Center (Washington, D.C.). Cells were maintained in Richter's Improved Minimal Essential Medium (IMEM) containing phenol red, supplemented with 5% fetal bovine serum, and cultured as previously described [10].

Preparation of glycosphingolipids

Total glycosphingolipids were isolated from cells by extraction, partition, and mild alkali treatment, and then fractionated by DEAE-Sephadex column chromatography into the four fractions of neutral glycolipids and mono-, di-, and tri- and tetrasialogangliosides, as described previously [18].

Quantification of neutral glycolipids and gangliosides

Total gangliosides were obtained from a DEAE-Sephadex column with 10 volumes of chloroform/methanol/2.0 M aqueous ammonium acetate (3:7:1) after neutral glycolipids were eluted and quantitated as previously described [19]. Ganglioside-bound sialic acids were measured by the 2-thiobarbituric acid method [19]. Sugars in total neutral glycolipids were quantified by a modified phenol-sulfuric acid method [20]. Amounts of each major ganglioside were calculated based on the percentage distribution of gangliosides obtained by scanning the thin-layer chromatogram (TLC) with a densitometer (Model CS-9300PC, Shimazu, Japan).

TLC analysis

After developing precoated thin-layer plates (HPTLC silica gel 60, EM Science) in the solvent system described in the text, the gangliosides were visualized by spraying with resorcinol reagent [21] followed by heating at 95° C. Neutral glycolipids were detected by heating after spraying with 20% sulfuric acid or anthrone-sulfuric acid reagent. Identification of glycosphingolipids was based on TLC mobilities compared to standards, specific enzymatic digestions, and whenever possible, by immunostaining of TLCs.

Detection of asialo-GM1 and GM1

Gangliosides were separated on HPTLC and the plates were fixed with 0.1% poly(isobutyl methacrylate) in chloroform and air dried [18]. Gangliosides were hydrolyzed for 3 h at room temperature *in situ* with *V. cholera* sialidase (40 mU/ml) and then washed once with PBS. After immersion in PBS containing 1% BSA for 20 min, the gangliosides were subsequently overlaid with rat anti-GA1 (1:250 dilution) for 1 h, biotinylated anti-rabbit IgG (1:250 dilution) for 1 h, and Vectastain ABC solution for 1 h, and then the peroxidase activity was detected with 4-chloro-1-naphthol/H_2O_2 solution [22].

In order to identify the minor CTB-binding monosialogangliosides, the sensitive overlay technique was used [18]. Briefly, after gangliosides were chromatographed on HPTLC and fixed, the chromatogram was overlaid with 1% BSA in PBS for 20 min, CTB (1:500 dilution) for 1 h, goat anti-CTB (1:500 dilution) for 1 h, and then with biotinylated anti-goat IgG (1:250 dilution) for 1 h. The bound toxin was detected by Vectastain ABC solution and 4-chloro-1-naphthol/H_2O_2 solution.

Enzymatic hydrolysis

15 nmol of gangliosides were treated with 0.16 unit of sialidase from *Clostridium perfringens* (Type V) in 200 µl of 0.05 M sodium acetate buffer (pH 5.1) at 37° C for 3 h and desalted with a Sep-Pak C18 cartridge [24]. α-neutral glycolipids (15 nmol) were hydrolyzed with 0.03 unit of β-galactosidase from *Streptococcus* 6646K in 200 ml of 0.05 M sodium

acetate buffer (pH 5.5) containing taurodeoxycholate (1 μg/μl) at 37° C for 3 h and desalted with a Sep-Pak C18 cartridge. Stepwise hydrolysis of neutral glycolipids of MCF-7 cells was carried out as follows. The lipid sample (22.5 nmol) was hydrolyzed with 0.08 unit of α-fucosidase from *Charonia lampas* in 300 μl of 0.05 M sodium acetate buffer (pH 4.9) containing taurodeoxycholate (1 μg/μl) at 37° C for 7 h and desalted with a Sep-Pak C18 cartridge. One-half of the sample was analyzed by TLC, and the other was subjected to β-galactosidase treatment as described above, desalted, and then analyzed by TLC.

DNA synthesis

Cells were cultured in estrogen-free, phenol red-free IMEM in the presence of 5% charcoal-stripped calf serum for 4 days and then changed into serum-free IMEM for 1 day. Prior to EGF treatment, cells were treated with GM3 overnight. After treatment with EGF for 1 day, cells were pulsed with [^{3}H]thymidine (1 μCi/ml) for 24 h. Incorporation of radioactivity into trichloroacetic acid-insoluble material was then measured [25]. Values are the means of triplicate determinations, and standard deviations were routinely less than 10% of the mean.

Results

Neutral glycolipids

MCF-7 cells contained more neutral glycolipids than MDA-MB-231 cells (Table 1). Figure 1 illustrates the neutral glycolipid compositions in these two cell lines. The two major neutral glycolipids in MDA-MB-231 and MCF-7 cells were indentified as CTH and globoside based on identical TLC migrations with the reference glycolipids in two different solvent systems. The doublet appearance of these components may be due to heterogeneity of ceramide composition. In addition, two other major neutral glycolipids were present in MCF-7 cells with mobilities similar to, but not exactly the same as asialo-GM1. That is, although the upper band migrated in the same position as asialo-GM1 in the neutral solvent system (Figure 1A), it showed a slightly faster mobility than asialo-GM1 in the ammonium hydroxide-containing solvent (Figure 1B). In agreement, both bands also were not recognized by anti-asialo-GM1 antibody (data not shown). Further experiments were carried out to attempt to identify these two bands. A lacto-series neutral glycolipid (nLc_6Cer) had a slightly larger Rf value in

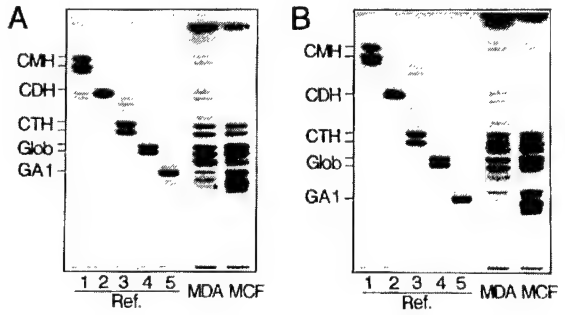

Figure 1. Neutral glycolipids of MDA-MB-231 and MCF-7 human breast cancer cells. Neutral glycolipids were separated by TLC with chloroform/methanol/0.2% $CaCl_2$ (60:35:8) in A and chloroform/methanol/2.5 N NH_4OH (60:35:8) in B, and visualized with 20% sulfuric acid. MDA, neutral glycolipids of MDA-MB-231 cells; MCF, neutral glycolipids of MCF-7 cells. Ref. (reference glycolipids): 1, CMH; 2, CDH; 3, CTH; 4, globoside; 5, asialo-GM1. * Nonglycolipid component which gives a yellowish band with anthrone-sulfuric acid reagent.

Table 1. Neutral glycolipids and gangliosides in MDA-MB-231 and MCF-7 cells

	Neutral glycolipids (μg galactose/1 × 10^7 cells)	Gangliosides (μg NeuAc/1 × 10^7 cells)
MDA-MB-231	5.1	2.7
MCF-7	10.4	0.67

Neutral glycolipids were determined by quantifying sugars in the neutral glycolipid fraction obtained from a DEAE-Sephadex column using the phenol-sulfuric acid method. Sialic acid (N-acetylneuraminic acid) contents in total gangliosides prepared by DEAE-Sephadex column chromatography were measured using the 2-thiobarbituric acid method.

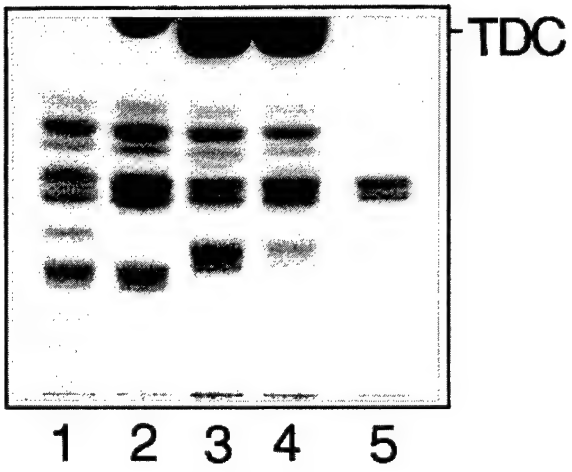

Figure 2. Exoglycosidase hydrolysis of neutral glycolipids of MCF-7 cells. Neutral glycolipids of MCF-7 cells (lane 1); neutral glycolipids hydrolyzed with β-galactosidase (lane 2), with α-fucosidase (lane 3), or with β-galactosidase after α-fucosidase treatment (lane 4); Glob, globoside standard (lane 5). The glycolipids were desalted with a Sep-Pak C18 cartridge after the glycosidase treatment and separated by TLC with chloroform/methanol/2.5 N NH_4OH (60:35:8). TDC, taurodeoxycholate. In the neutral glycolipids of MCF-7 cells (lane 1), the bands of globoside and glycolipid with lower Rf values than globoside showed irregular shapes which could be due to the effect of contaminating salt.

the neutral solvent system and lower mobility in the ammonium hydroxide-containing solvent than the unidentified two bands. Forssman glycolipid, a derivative of globoside with α-N-acetylgalactosaminyl extension, migrated faster than the two bands. As a glycosphingolipid with a galactosyl extension has lower mobility on TLC than the same glycosphingolipid with an N-acetylgalactosaminyl extension [26], the possibility that these bands may be galactosylgloboside was raised. Furthermore, the presence of galactosylgloboside (IV^3Galβ-Gb_4Cer) and fucosylgalactosylgloboside (IV^3(Fucα1-2--Galβ)-Cb_4Cer) has been reported in neutral glycolipids of MCF-7 cells [16, 17], although they were shown to be rather minor components of the neutral glycolipids [17]. To examine the possibility that these two glycolipids are derivatives of globoside, the neutral glycolipids of MCF-7 cells were hydrolyzed with β-galactosidase and α-fucosidase. As shown in Figure 2, the upper band of these two unidentified bands was hydrolyzed with β-galactosidase, while the lower band was resistant (lane 2). After the β-galactosidase treatment, no additional band appeared in the neutral glycolipid fraction. However, the lower band was susceptible to α-fucosidase digestion and produced a new band with similar mobility to the upper band (lane 3). This product was also susceptible to β-galactosidase digestion and the amount of globoside increased after β-galactosidase treatment (Figure 2, lane 4). From these results, the upper and the lower band of the two unidentified bands in MCF-7 cells were tentatively identified as galactosylgloboside and fucosylgalactosylgloboside, respectively.

Gangliosides

In contrast to the relative amounts of neutral glycolipids, MDA-MB-231 cells contained about 4 fold more ganglioside-bound sialic acid than MCF-7 cells (Table 1). The gangliosides were fractionated on DEAE-Sephadex columns and the gangliosides in these cell lines were recovered in mono- and disialoganglioside fractions. The TLC patterns of mono- and disialogangliosides in MDA-MB-231 and MCF-7 cells are shown in Figure 3. Components 1, 2, and 3 in the monosialoganglioside fraction from MDA-MB-231 and MCF-7 cells had identical mobilities as GM3, GM2, and GM1 in three different solvent systems. Component 5 in MDA-MB-231 cells migrated slightly faster than IV^3NeuAcα-nLc4Cer in both the neutral solvent system (Figure 3A) and the chloroform-methanol-ammonium hydroxide system (Figure 3B), but appeared to migrate slower than IV^3NeuAcα-nLc4Cer in the propanol-ammonium hydroxide solvent system (Figure 3C). Component 4 in the disialoganglioside fractions from both cell lines migrated in the same position as standard GD1a in all three solvent systems.

Gangliosides were treated with sialidase to further confirm the structure assignments. Component 1 was hydrolyzed with sialidase and produced the expected product CDH (Figure 4A). Components 2 and 3, which had identical Rf values to those of standard GM2 and GM1, were confirmed to be sialidase-stable. Component 5 in MDA-MB-231 cells was susceptible to sialidase. A glycolipid corre-

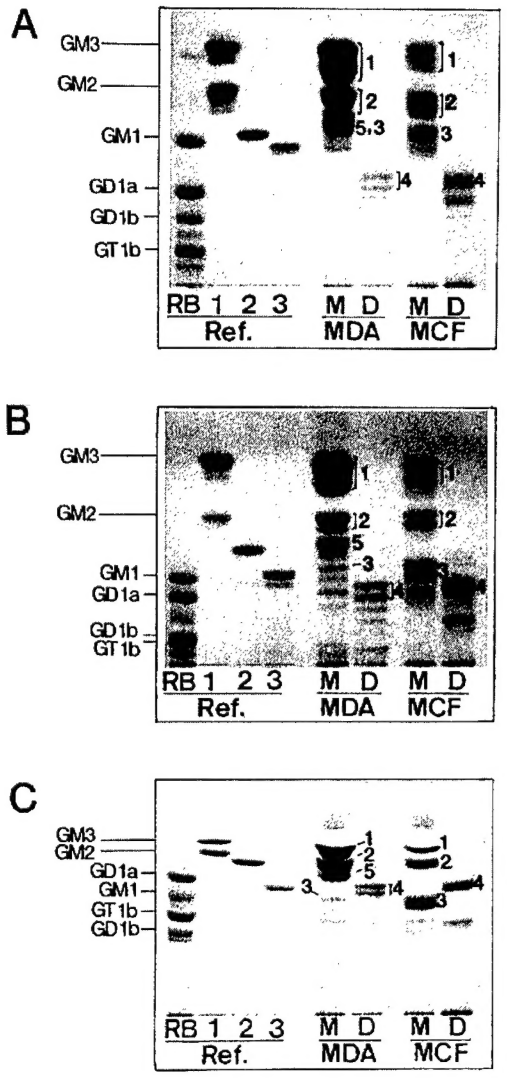

Figure 3. Gangliosides of MDA-MB-231 and MCF-7 cells. Gangliosides were separated by TLC using chloroform/methanol/0.2% $CaCl_2$ (60:40:9) in A, chloroform/methanol/2.5 N NH_4OH (60:40:9) in B, and *n*-propanol/28% NH_4OH/water (75:5:25) in C, and visualized with resorcinol reagent. M, monosialogangliosides, and D, disialogangliosides, obtained from MDA-MB-231 cells (MDA) or MCF-7 cells (MCF). Ref.; RB, total gangliosides from rat brain; 1, mixture of GM3 (NeuAc) and GM2 (NeuAc); 2, sialylparagloboside (IV^3NeuAcα-nLc_4Cer); 3, sialylparagloboside (IV^6NeuAcα-nLc_4Cer).

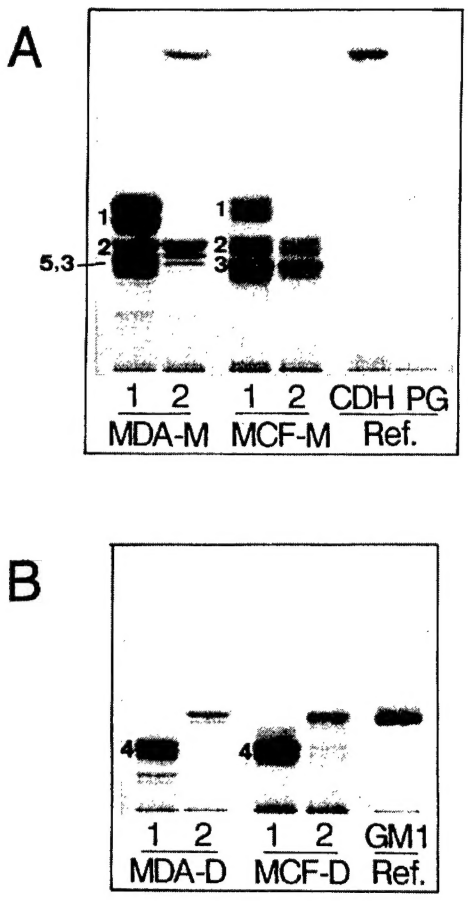

Figure 4. Sialidase hydrolysis of mono- (A) and disialogangliosides (B). MDA-M and MCF-M, monosialogangliosides from MDA-MB-231 and MCF-7 cell line; MDA-D and MCF-D, disialogangliosides from MDA-MB-231 and MCF-7 cells; Lane 1, before; and lane 2, after hydrolysis; Ref. PG, paragloboside. The glycosphingolipids were separated by TLC using chloroform/methanol/0.2% $CaCl_2$ (60:35:8) and visualized with resorcinol reagent. Standard neutral glycolipids and hydrolysis products were detected as resorcinol-negative, yellow bands.

sponding to paragloboside was not detected after sialidase hydrolysis. Instead, two neutral glycolipid bands were produced with slightly greater mobility than paragloboside and with similar mobility to globoside. Component 4 in the disialoganglioside fractions from both cell lines was hydrolyzed with sialidase and gave the hydrolysis product corresponding to GM1.

From these results, components 1, 2, 3, and 4 were identified as ganglio-series gangliosides GM3, GM2, GM1 and GD1a, respectively. Component 5 appeared to have a sialic acid residue linked to the terminal sugar of tetraose backbone which has a Rf value similar to that of globoside.

A prominent difference between the gangliosides of these two cell lines was in the amount of GM3. As shown in Table 2, GM3 was the major ganglioside in MDA-MB-231 cells and there was 18 fold

more than in MCF-7 cells. In contrast, the most predominant ganglioside in MCF-7 cells was GM1 which was present at 3-fold higher levels than in MDA-MB-231 cells. These differences in ganglioside levels were further confirmed by sensitive immunostaining with CTB (data not shown).

Inhibition of DNA synthesis induced by EGF after insertion of exogenous ganglioside GM3

In agreement with a previous report that EGF is a mitogen for MCF-7 but not for MDA-MB-231 cells [28], we have found that EGF stimulates proliferation of MCF-7 cells as measured by [^{3}H]thymidine incorporation. A mitogenic effect was observed at a concentration of EGF as low as 0.1 ng/ml and maximum stimulation was achieved at 10 ng/ml (data not shown). The level of membrane-associated gangliosides can be manipulated by the functional insertion of exogenous gangliosides [1, 2, 25]. MCF-7 cells readily incorporated exogenously administered GM3 ganglioside. Such treatment significantly inhibited EGF-induced proliferation (Table 3).

Discussion

The present study demonstrated that there is a marked difference in composition of neutral glycolipids and gangliosides of MDA-MB-231 and MCF-7 human breast cancer cell lines. All of the abundant neutral glycolipids in both cell lines belonged to the globo-series of glycolipids, whereas the majority of the gangliosides were ganglio-series gangliosides. Two neutral glycolipids in MCF-7 cells which did not correspond to any standard glycolipids examined were tentatively identified as galactosylgloboside and fucosylgalactosylgloboside based on the results of exoglycosidase hydrolyses. The structures of galactosylgloboside, IV^{3}Galβ-Gb$_4$Cer, and fucosylgalactosylgloboside, IV^3(Fuc α1-2Galβ)-Gb$_4$Cer, were originally elucidated in human teratocarcinoma cells as rare examples of extended globo-series glycolipids in human cells and tissues [27]. The large amounts of these two glycolipids in MCF-7 cells are remarkable, since these glycolipids were previously characterized as minor components of MCF-7 cells using monoclonal antibodies derived from mice immunized with MCF-7 cells or antibodies from patients with primary lung cancer [16, 17].

The most striking difference in the glycosphingolipids of these cell lines was the enormous amount of GM3 in MDA-MB-231 cells. GM3 added exogeneously inhibits tyrosine phosphorylation of the epidermal growth factor receptor in many cell lines, including human epidermal carcinoma cells [6]. It has been shown that GM3 binds specifically to the EGF receptor [7] and such association probably inhibits EGF-mediated growth by preventing dimerization of EGF receptor monomers. It has been proposed that ganglioside inhibition of receptor dimerization may be a novel mechanism for regulating and coordinating several trophic factor-mediated cell functions [8]. In contrast to MCF-7 cells, which contain EGF receptors and whose growth is

Table 2. Composition of the major gangliosides in MDA-MB-231 and MCF-7 cells

	GM3	GM2	GM1	GD1a	Others
	(μg NeuAc/1 × 10^7 cells)				
MDA-MB-231	1.59	0.41	0.062	0.27	0.40
MCF-7	0.087	0.12	0.17	0.13	0.15

Ganglioside concentrations were calculated based on the percentage distribution obtained by densitometric scanning of TLCs and the total amount of NeuAc in the ganglioside fraction.

Table 3. Effects of GM3 pretreatment on EGF-induced proliferation

Treatment	[^{3}H]Thymidine incorporation (cpm/well)
None	11100 ± 600
EGF	14700 ± 500
GM3	10400 ± 800
GM3 + EGF	12200 ± 1300

Cells were incubated overnight at 37° C in serum-free, phenol red-free IMEM, in the presence or absence of GM3 (50 μM). Cells were then exposed to EGF (10 ng/ml) and [^{3}H]thymidine incorporation was determined as described in Materials and methods. Values are means ± SD of triplicate determinations from a representative experiment of two experiments performed.

stimulated by EGF [28], MDA-MB-231 cells, although overexpressing EGF receptors, are unresponsive to EGF. In agreement, pretreatment of MCF-7 cells with GM3 decreased the stimulatory effect of EGF on cell proliferation. Thus, the high level of GM3 in MDA-MB-231 cells may abrogate EGF receptor functions in these cells. However, tumor cell growth, invasion, and metastasis are complex processes that depend on many other factors and glycosphingolipid composition may be one of the contributors.

CTH, also known as Gb3/CD77, is a glycolipid which is specifically expressed on two different B-cell populations, Burkitt's lymphoma and a subset of tonsillar B-lymphocytes located in germinal centers. Both of these B cells have recently been shown to readily undergo programmed cell death (apoptosis). Verotoxin, a shiga-like toxin which binds to the carbohydrate moiety of Gb3/CD77, induced apoptosis of Burkitt's lymphoma cells [29] and is the first example of a glycolipid antigen which transduces signals leading to apoptosis. CTH was also identified as one of the major neutral glycolipids in MDA-MB-231 and MCF-7 cells. As apoptosis is considered to be a cellular component which affects cancer progression, studies on the contribution of these glycosphingolipids to apoptosis may give new insights into the role of glycosphingolipids in cancer. In the present study, we found that ganglioside GM1 is the major ganglioside in MCF-7 cells. Recently, we demonstrated that CTB differentially induces programmed cell death in MCF-7 but not in MDA-MB-231 cells [30]. These results suggest that ganglioside GM1 may play a role not only in cell growth regulation but also in apoptosis.

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